THE INFLUENCE OF ANIMAL PROTEIN FACTOR SUPPLEMENTS AND ANTIBIOTICS ON THE INCIDENCE AND SEVERITY OF WHITE FEATHERS IN TURKEYS

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TWO FIGURES

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Fritz et al. ('46) showed that lysine was necessary for the prevention of a white bar condition in the flight feathers of bronze poults. Grau et al. ('46) found a lysine requirement for poults of 1.3%, while German et al. ('49) obtained normal feather pigmentation with 1.2% lysine in the diet. Slinger et al. ('49) and Gartley et al. ('50) found that sunflower seed oil meal was seriously deficient in lysine for the poult when used to supply most of the supplementary protein in either a 24 or 28% protein diet. The results of the latter workers suggested a higher dietary lysine requirement for normal feather pigmentation than for optimum growth in poults.

Several workers have reported that certain animal protein factor (APF) supplements cause a marked increase in the growth of poults (McGinnis et al., '49; Singsen and Matterson, '49; Slinger, '50; and others). Stokstad and Jukes ('50) have shown that aureomycin, at low levels, causes growth stimulation in poults, while McGinnis ('50) has reported that penicillin, aureomycin, terramycin and streptomycin produce a substantial growth increase in this species.

Newly hatched broad breasted bronze poults were used in all of the tests to be reported. The birds were vent-sexed and

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allotted to groups on the basis of sex and weight. The protein ($N \times 6.25$) content of the basal diets was determined and lysine analyses were made according to the method of Henderson and Snell ('48).

EXPERIMENTAL

Experiment 1

This experiment was undertaken to determine the effect of an APF supplement on growth and feather pigmentation when added to diets containing either soybean oil meal or sunflower seed oil meal as the sole supplementary protein. The experiment was comprised of three groups of 25 and one group of 38 poults. The birds were maintained in electrically heated battery brooders with raised wire floors. The experimental diets and tap water were supplied ad libitum for the 4 weeks' duration of the trial. The percentage composition of the diet for group 1 was as follows: corn 17, wheat 17, oat groats 5, dehydrated alfalfa meal 1, soybean oil meal (solvent) 51, soybean oil 1.75, limestone 1.5, steamed bone meal 5.25, iodized salt 0.5, and fish oil (400 D–2,400 A) 1.0. In addition 9 gm of manganese sulfate (technical), 0.2 gm of riboflavin and 1 gm of niacin were added to each 100 lb. of diet. The diet for group 3 differed from that of group 1 only in that the former contained 52.75% of sunflower seed oil meal (expeller), replacing the soybean oil meal and soybean oil.

The results of experiment 1 are shown in table 1. Growth and feed efficiency were improved markedly by the addition of the APF supplement [1] to the soybean oil meal diet, while little or no improvement resulted from such addition to the sunflower seed oil meal diet. All birds in groups 3 and 4 exhibited white feathers. Each bird was assigned a white feather score of 1 to 4 depending upon the severity of the condition. The averages of these values are presented in table 1. It will be noted that the addition of the APF supplement to the sunflower seed oil meal diet increased the severity of the white feather condition. It is of interest to note also that, while many cases of broken wing feathers were present in group 1

[1] Lederle no. 5.

and particularly in group 3, the addition of the APF supplement completely prevented this condition in the case of group 2 and markedly decreased the incidence in group 4. The severe white feather condition resulting from the addition of the APF supplement to the sunflower seed oil meal diet is illustrated in figure 1.

TABLE 1

The effect of an APF supplement on growth and white feathers when added to a soybean oil meal and a sunflower seed oil meal diet

GROUP NO.	TREATMENT	PROTEIN (N × 6.25)	LYSINE	WEIGHT AT 4 WEEKS OF AGE			LB. FEED/LB. GAIN	WHITE FEATHER SCORE
				Males	Females	Unweighted mean		
		%	%	*gm*	*gm*	*gm*		
1	Soybean oil meal	29.0	1.8	411 (10)[1]	336 (11)	374 (21)	2.18	0
2	As 1 + 0.75% Lederle APF no. 5			644 (19)	577 (19)	611 (38)	1.75	0
3	Sunflower seed oil meal	29.7	0.9	237 (6)	215 (12)	226 (18)	2.06	1.67
4	As 3 + 0.75% Lederle APF no. 5			266 (10)	241 (13)	254 (23)	2.32	3.55

[1] Figures in parentheses represent the number of birds surviving.

Experiment 2

This experiment was designed to compare the performance of poults fed 20 and 28% protein diets when both diets contained an APF supplement. These diets were considered just adequate and high in lysine content, respectively, on the basis of normal dietary requirements. Group 1 consisted of three

replicate pens of 50 birds and group 2 of 6 replicate pens of 50 birds each. The birds were reared on litter in similar pens of two brooder houses. The percentage composition of the 20% protein ration was as follows: yellow corn 30, wheat 17, wheat shorts 15, oat groats 5, oats 5, dehydrated alfalfa meal 2, fish meal 5, meat meal 5, dried buttermilk 5, soybean oil meal (solvent) 7, limestone 1.5, steamed bone meal 1.25, iodized salt

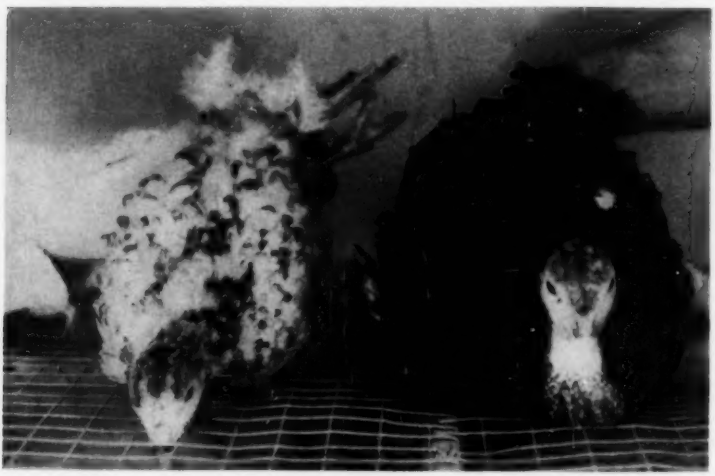

Fig. 1 Showing severe white feather syndrome caused by feeding a lysine-deficient diet plus APF. Bird on left fed the sunflower seed oil meal diet plus APF; bird on right, the soybean oil meal diet plus APF. Age 7 weeks.

0.25, and fish oil (400 D–2,400 A) 1. In addition 11.3 gm of manganese sulfate (technical), 0.2 gm of riboflavin, 1 gm of niacin, and 22.7 gm of a different APF supplement [2] were added to each 100 lb. of diet. The 28% protein diet was the same as the 20% diet excepting that in the former case 26% additional soybean oil meal (solvent) was added, making a total of 33%, at the expense of 15% of wheat shorts and 11% of yellow corn.

[2] Merck experimental APF supplement no. 8.

The results of experiment 2 are shown in table 2. An outbreak of coccidiosis was responsible for most of the mortality in the group being fed the 20% protein diet. The disease was not as severe in the group fed 28% protein but no evidence existed to suggest that the diets being used were responsible for this difference. The presence of the disease is not believed to have altered the results materially. Growth and feed efficiency were considerably better with the 28 than the 20% protein diet.

A high incidence of white feathers was present in group 1, while practically no such cases were noted in group 2. The diet fed to group 1 contained 1.2% lysine, a level which Ger-

TABLE 2

The effect of protein level on growth and the incidence of white feathers in the presence of an APF supplement

GROUP NO.	PROTEIN (N × 6.25)	LYSINE	WEIGHT AT 4 WEEKS OF AGE			LB. FEED/ LB. GAIN	PERCENTAGE INCIDENCE OF WHITE FEATHERS
			Males	Females	Unweighted mean		
	%	%	*gm*	*gm*	*gm*		
1	20.2	1.2	333 (59)[1]	291 (61)	312 (120)	2.21	51.7
2	28.4	1.9	588 (148)	515 (151)	552 (299)	1.82	1.7

[1] Figures in parentheses represent the number of birds surviving.

man et al. ('49) and others have shown to be adequate for normal feather pigmentation. Furthermore, a 20% protein diet similar to that used here, but not containing APF, has resulted in normal feather pigmentation in past studies in our laboratories (unpublished). The appearance of typical birds fed the 28 and 20% diets is shown in figure 2.

Experiment 3

Experiment 3 was an attempt to confirm the suggestion from experiment 2 that the addition of an APF supplement to a diet just adequate in lysine greatly increased the incidence of white feathers. Two antibiotics were also tested for similar

behavior. Eight groups, each consisting of 19 males and 17 females, were employed in this work. The birds were housed in electrically heated battery brooders with raised wire floors. The experimental diets and tap water were before the birds at all times during the 22 days' duration of the trial. The diets fed to groups 1 and 5 were of the same composition as those used in experiment 2 except that for the present work

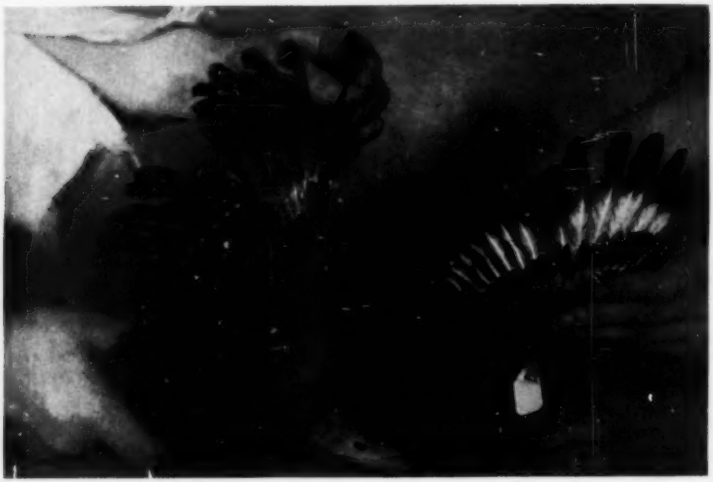

Fig. 2 Representative birds from groups fed diets containing 28 and 20% protein supplemented with APF. Note difference in size, and white feathers of bird fed 20% protein diet. Age 17 days.

the APF supplement was omitted. Penicillin G potassium and aureomycin hydrochloride were added at the rate of 25 p.p.m. of the salts to the diets for groups 2 and 6, and 3 and 7, respectively. An APF supplement [3] was added to the diets for groups 4 and 8 at the rate of 22.7 gm per 100 lb.

The results of this experiment are presented in table 3. Considerable mortality occurred in groups 1 and 5. While the cause of this mortality is unknown, many of the birds in these

[3] See footnote 2, page 348.

groups were unthrifty in appearance and showed signs of whitish diarrhea. The addition of the antibiotics or the APF supplement appeared to correct these symptoms and elimi-

TABLE 3

The effect of antibiotics and an APF supplement on growth and white feathers with different levels of protein

GROUP NO.	TREATMENT	PROTEIN (N × 6.25)	LYSINE	WEIGHT AT 22 DAYS			LB. FEED/ LB. GAIN	PERCENTAGE INCIDENCE OF WHITE FEATHERS
				Males	Females	Unweighted mean		
		%	%	*gm*	*gm*	*gm*		
1	28% protein	28.4	1.9	218 (16)[1]	224 (12)	221 (28)	4.30	3.3
2	As 1 + penicillin			410 (17)	354 (17)	382 (34)	1.93	2.9
3	As 1 + aureomycin			412 (19)	358 (17)	385 (36)	1.77	nil
4	As 1 + Merck APF no. 8			390 (18)	315 (16)	353 (34)	1.90	8.8
5	20% protein	20.2	1.2	182 (13)	186 (12)	184 (25)	2.57	nil
6	As 5 + penicillin			272 (19)	229 (17)	251 (36)	2.28	77.8
7	As 5 + aureomycin			269 (19)	211 (16)	240 (35)	2.56	82.9
8	As 5 + Merck APF no. 8			231 (18)	201 (17)	216 (35)	2.59	94.3

[1] Figures in parentheses represent the number of birds surviving.

nated mortality. The data indicate that the antibiotics and APF supplement resulted in a much greater response in growth and feed efficiency when added to the 28% than to the

20% protein diet. The response to these materials was also greater in males than in females. The somewhat poorer growth resulting from the APF supplement than from the antibiotics suggests that under these conditions the concentration of antibiotic added from the former source may have been insufficient.

Supplementing the 20% protein diet with penicillin, aureomycin or the APF concentrate caused a very high incidence of white feathers. The APF-supplemented groups showed a somewhat higher incidence of this disorder than the groups being fed penicillin or aureomycin at the same protein level.

DISCUSSION

Kratzer et al. ('50) showed that the level of lysine required to prevent pigmentation failure increases as the protein level increases. According to the data of these workers, 1.0% of lysine was sufficient to prevent white feathers with a diet containing 20% of protein. The increase in severity of white feathers with a lysine-deficient diet and the production of the disorder with a 20% protein diet containing 1.2% of lysine, by the addition of an APF supplement or antibiotics, suggest that these materials increase the dietary level of lysine required for normal feather pigmentation. The fact that the APF supplements or antibiotics resulted simultaneously in an increase in growth and a higher incidence of white feathers with diets containing 1.2% of lysine suggests that the growth requirement for this amino acid takes precedence over the feather pigmentation requirement. This is further evidence in support of the work of Gartley et al. ('50) suggesting that white feathers are a more sensitive criterion of lysine deficiency than is growth.

In experiment 3, the groups fed the APF supplement showed a somewhat higher incidence of white feathers than the groups being fed penicillin or aureomycin at the same level of protein. This would suggest that the APF supplement used contained a factor other than an antibiotic which also increased the lysine requirement to prevent white feathers. Whether

vitamin B_{12} is the factor concerned cannot be decided from this work.

The much greater response to APF and antibiotics using a 28 rather than a 20% protein diet may indicate that to reach the maximum potential growth under the influence of these materials the requirement for other amino acids, as well as lysine, is also increased. It seems possible that the protein requirement of poults fed diets containing APF or antibiotics may be in excess of the 28 to 30% found by Scott et al. ('48) to be necessary in the absence of these materials. Moore et al. ('46) showed that orally administered streptomycin brought about a marked reduction in the numbers of coliform bacteria in the feces of chicks, while Groschke and Evans ('50) reported that the injection of aureomycin into chicks failed to increase growth. In view of these findings, the latter workers suggest that the growth effect from antibiotics is mediated in some manner through a changed intestinal microflora. A higher protein requirement in the presence of APF or antibiotics may be explained, then, on the basis of increased availability of ingested nutrients or intestinal synthesis of a growth factor or factors. Since the 28% protein diet employed is considered to have been adequate in respect to known nutrients, it may be that an as yet unrecognized factor, or factors, is being synthesized.

SUMMARY

The inclusion of an APF supplement in a soybean oil meal diet resulted in marked improvement in growth and feed efficiency. When this APF supplement was added to a sunflower seed oil meal diet, containing 0.9% of lysine, little or no response occurred in growth and feed efficiency. None of the birds fed the soybean oil meal diets developed white feathers, while all the birds fed the sunflower seed oil meal diets showed this condition. The addition of the APF supplement to the sunflower seed oil meal diet increased the severity of the white feather syndrome.

In a further experiment, groups of birds were fed diets containing 20 and 28% protein supplemented with an APF concentrate. Although the 20% protein diet contained 1.2% of lysine, over 50% of the birds developed the white bar condition of the flight feathers. Only an insignificant number of white feathers appeared in the group fed the 28% protein diet with 1.9% of lysine.

In a final experiment, groups of birds were fed diets containing 20 and 28% protein with and without penicillin, aureomycin and an APF supplement. The antibiotics and APF supplement resulted in a much greater response in growth and feed efficiency when added to the 28% than to the 20% protein diet. Supplementing the diet containing 20% protein and 1.2% lysine with penicillin, aureomycin or the APF concentrate, resulted in a high incidence of white feathers.

The results indicate that certain APF concentrates, penicillin and aureomycin increase the lysine requirement of the poult for normal feather pigmentation. It is also suggested that these materials increase the requirement of this species for other amino acids. Possible explanations of these effects may be that, under the influence of certain APF supplements and antibiotics, the availability of ingested nutrients is increased or an unknown growth factor, or factors, is synthesized in the intestinal tract.

ACKNOWLEDGMENTS

The APF supplements used in this study were generously supplied by Merck and Company, Limited, Montreal, and the Lederle Laboratories, Pearl River, New York. The Merck APF no. 8 was represented as containing 12.5 mg vitamin B_{12} activity and 2.5 gm procaine penicillin per pound. The Lederle APF supplement no. 5 was represented as containing between 1.5 and 2.0 mg vitamin B_{12} and 1.0 to 2.0 gm of aureomycin per pound. The penicillin G potassium was supplied through the courtesy of Chas. Pfizer and Company, Inc., Brooklyn, New York, and the aureomycin hydrochloride through the courtesy of the Lederle Laboratories.

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STUDIES ON THE CHRONIC ORAL TOXICITY OF COTTONSEED MEAL AND COTTONSEED PIGMENT GLANDS

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The high nutritive value of cottonseed meal and its use as a source of protein in livestock feeds have been well established. However, there appears to be some question whether cooking is essential to obtain a meal that is safe, readily digestible, and biologically available. Deleterious physiological effects of uncooked cottonseed meal have been attributed by various investigators to the presence of gossypol, which occurs in varying amounts as part of the pigment-gland fraction of cottonseeds. For a comprehensive review of the literature, the reader is referred to the monograph by Hale and Lyman ('48) on the use of cottonseed as a source of animal feedstuffs. Suggestions for the detoxification of the oil-free meal by cooking, by autoclaving, by air oxidation, or by the addition of iron salts have been made by Gallup ('27a, b, '28), and by Olcott ('48).

As part of a program of study on the utilization of cottonseed meal and its products, investigators at the Southern Regional Research Laboratory (Boatner and Hall, '46; Boatner, Hall, Rollins and Castillon, '47; Vix et al., '47) have shown that pigment glands, which contain most of the pigment of cottonseeds, are resistant to the action of most solvents and are mechanically very strong. These same investigators have devised a process, referred to as the "gland flotation

process," whereby the pigment glands can be readily removed from the cottonseed meal in an unaltered condition, thus making available pigment glands and gland-free cottonseed meal in quantities sufficient for physiological testing.

Recently it has been reported by Groschke, Rubin and Bird ('47) and by Boatner et al. ('48) that uncooked gland-free cottonseed meal and cooked hydraulic-pressed cottonseed meal supported the growth of chicks and had no effect on hatchability, while the feeding of cottonseed pigment glands or uncooked hexane-extracted cottonseed meal containing intact pigment glands had the same detrimental effect on growth as the feeding of raw cottonseed meal containing an equivalent amount of cottonseed pigment glands. It was, therefore, concluded by Boatner et al. that all of the physiologically deleterious components of cottonseed are segregated in the pigment glands.

Studies in this laboratory have been concerned with a comparison of the toxicity of: (a) gland-free cottonseed meal and cottonseed pigment glands as obtained by the "gland flotation process" and (b), cottonseed meal prepared by hexane extraction.

Two samples of gland-free meal and two samples of pigment glands prepared by the "gland flotation process" described by Vix et al. ('47) and one sample of hexane-extracted meal were used in these studies. The analytical data for the gossypol content of the samples studied are presented in table 1. The samples and analytical data were kindly supplied by the Southern Regional Research Laboratory of this Bureau.

EXPERIMENTAL

Chronic toxicity

Young weanling male albino rats from our stock colony weighing 47 to 50 gm each were placed in cages in groups of 5. A commercial dog food [1] served as the control and basic ration in all experiments. The various supplements under

[1] Purina.

investigation were added to the basic ration in the concentrations listed in tables 2 and 3 by thoroughly mixing the supplements with the basic diet. In each case the more concentrated diet was prepared first by adding the calculated amount of test material. Other test diets were prepared by suitable dilution of the more concentrated diet with the basic diet. Free access to food and water was permitted at all times. Weekly records were kept of food consumption, body weight, and the general appearance of all rats. Periodic hematological studies, consisting of erythrocyte and leukocyte counts and measurement of hemoglobin concentrations, were made. At

TABLE 1

Description of cottonseed meal and pigment glands used[1]

	DESCRIPTION	GOSSYPOL
		%
CSM	Gland-free meal	0.057
CPG	Pigment glands	37.5
H	Hexane-extracted meal	1.1
G	Gland-free meal	0.076
PG	Pigment glands	36.5

[1] Samples and analytical data were supplied by the Southern Regional Research Laboratory of this Bureau.

the termination of the feeding period all of the surviving rats were weighed, killed with ether, exsanguinated, and carefully autopsied. All visceral organs were removed, fixed in 10% formalin, and saved for histopathological study. Hematoxylin-eosin stained sections of the following organs were prepared and submitted to a pathologist for study—thyroid, lung, heart, adrenals, kidney, liver, spleen, stomach, intestine, pancreas, urinary bladder, and testes.

Paired feeding

In order to determine more definitely the extent of growth inhibition, in rats on the high dietary supplements of cotton-

seed pigment glands, which could be accounted for by anorexia alone, paired-feeding studies according to the procedure of Mitchell and Beadles ('30) were made. Litter mates of both sexes having the same initial body weight were used. Food consumption of each rat receiving the supplements was accurately determined each day. To the pair-fed control rats an amount of basic diet was offered equivalent to that consumed by the respective supplement-fed rats. The body weights of each rat on the supplements as well as those of the pair-fed controls were recorded each day.

Five pairs of male and 5 pairs of female rats were used for studies with the diet containing 0.256% cottonseed pigment glands, and a similar number of pairs of rats were used with the diet containing 0.512% cottonseed pigment glands. In order to determine further whether anorexia was due to cottonseed pigment glands alone or to the principal active constituent of the glands (gossypol), paired-feeding studies were conducted with 5 pairs of male and 5 pairs of female rats on a diet containing 0.192% gossypol, since it had been reported by Zucker and Zucker ('47) that purified gossypol has an appetite-depressing effect of possible clinical importance in the treatment of obesity.

Acute and subacute toxicity produced by feeding via a stomach tube

The oral toxicities of cottonseed pigment glands and gossypol were determined in young albino rats of both sexes from our stock colony, weighing 100 to 150 gm each. Solutions of the respective substances were made with water except for gossypol, which was solubilized with an equal weight of methyl glucamine [2] in water.

For testing the acute oral toxicity of a single dose, 40 rats were fed cottonseed pigment glands in dosages ranging from 2,000 to 4,000 mg/kg in multiples of 500 mg. For testing gossypol, 9 rats were fed 500 mg/kg, and two were used for

[2] Methyl glucamine was kindly supplied by the Abbott Laboratories.

each of the following dosage levels: 200, 400, 800, 1,600, and 3,200 mg/kg.

For determining the subacute oral toxicity of cottonseed pigment glands, 28 rats were used; three repeated doses of 1,000 mg/kg were given to 14 rats at 7-day intervals, and a similar number received 2,000 mg/kg. For gossypol, 9 rats were used for 250 mg/kg and 9 for 500 mg/kg; the dosage was repeated thrice at 5-day intervals.

RESULTS

On the basis of the data obtained, it is quite apparent that rats on the diets containing as much as 32% gland-free cottonseed meal (CSM) in the diet grew as well as the controls on the basic diet alone (table 2). Thus it appears that the feeding of gland-free cottonseed meal containing 0.076% or less gossypol or gossypol-like materials resulted in no toxicity or inhibition in growth. Further evidence that gland-free meal is non-toxic is presented in table 3, where a comparison is made between the growth of rats on a different sample of gland-free cottonseed meal (G) and rats on hexane-extracted cottonseed meal (H). From the data on growth, it is clearly evident that rats on the diet containing 15% hexane-extracted cottonseed meal (equivalent to approximately 0.16% gossypol) did not grow as well as the animals on the diet containing 30% gland-free meal. No inhibition in growth was observed in rats on 3.75 or 7.5% hexane-extracted cottonseed meal.

Rats fed one sample of cottonseed pigment glands (CPG) at 0.256% of the ration (equivalent to approximately 0.096% gossypol) showed a definite inhibition of growth (table 2). Those fed the second sample (PG) showed no inhibition in growth at the same level of intake, but at 0.512% of the ration (equivalent to approximately 0.18% gossypol) a similar inhibition in growth occurred (table 3). On the basis of the studies represented by these two sets of data, it is quite apparent that a difference in toxicity exists. It is doubtful that the toxicity can be ascribed entirely to the gossypol content, since the difference in gossypol content of the two samples of

TABLE 2

Summary of data on continued feeding of cottonseed pigment glands (CPG) and defatted depigmented cottonseed meal (CSM) to male rats. (Time: approximately 185 days)

SUPPLEMENT	NO. OF RATS		MEAN WEIGHT			E_D [2]	FOOD CONSUMPTION
	At start	At end	At start	At end	Increase [1]		
			gm	*gm*	*gm*		*gm/rat/day*
Control	5	5	47.0	295.4	248.4 ± 6.07		13.7
8% CSM	5	5	48.0	287.2	239.2 ± 8.71	10.62	13.4
16% CSM	5	5	48.8	275.8	227.0 ± 10.55	12.19	13.1
32% CSM	5	5	49.0	276.6	227.0 ± 5.64	8.29	13.3
Control	5	5	50.4	307.6	257.2 ± 7.99		13.8
0.001% CPG	5	5	49.0	270.0	221.0 ± 18.84	20.47	13.2
0.002% CPG	5	4	50.2	299.2	249.0 ± 8.93	11.99	13.6
0.004% CPG	5	5	50.0	295.4	245.4 ± 6.44	10.26	13.6
0.008% CPG	4	4	50.4	295.2	244.8 ± 5.85	9.90	13.7
0.016% CPG	5	5	50.4	292.4	242.0 ± 10.76	13.40	13.6
0.032% CPG	5	5	49.6	285.4	235.8 ± 8.07	11.36	13.3
0.064% CPG	5	5	49.4	277.2	227.8 ± 9.04	12.07	13.3
0.128% CPG	5	5	49.2	291.0	241.8 ± 9.46	12.37	13.6
0.256% CPG	5	5	49.4	226.6	177.2 ± 9.51	12.42	12.0
0.512% CPG	5	4	49.2	189.2	140.0 ± 6.96	10.59	12.3

[1] Standard error of the mean, $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

[2] E_D = standard error of the difference between means.

TABLE 3

Summary of data on continued feeding of cottonseed pigment glands (PG), defatted depigmented cottonseed meal (G), and hexane-extracted cottonseed meal (H) to male rats. (Time: approximately 195 days)

SUPPLEMENT	NO. OF RATS		MEAN WEIGHT			E_D [2]	FOOD CONSUMPTION
	At start	At end	At start	At end	Increase [1]		
			gm	*gm*	*gm*		*gm/rat/day*
Control	5	5	41.4	292.8	251.4 ± 16.25	....	14.9
0.032% PG	5	5	41.0	282.8	241.8 ± 2.09	16.38	14.3
0.064% PG	5	5	42.0	310.4	268.4 ± 5.72	17.23	14.8
0.128% PG	5	5	42.2	316.2	274.0 ± 12.28	20.37	14.8
0.256% PG	5	5	42.6	297.8	255.2 ± 9.40	18.77	14.7
0.512% PG	5	1	...	....	....	....	...
Control	5	5	42.0	299.8	257.8 ± 14.29	....	14.3
7.5% G	5	5	42.4	308.6	266.2 ± 2.90	14.56	14.7
15% G	5	5	41.2	300.4	259.2 ± 9.73	17.26	14.2
30% G	5	5	40.6	294.2	253.6 ± 14.01	20.00	13.3
3.75% H	5	5	41.6	305.8	264.2 ± 9.22	16.99	14.6
7.5% H	5	5	42.2	291.4	249.2 ± 14.29	20.19	14.5
15% H	5	2	...	....	....	....	...

[1] Standard error of the mean, $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

[2] E_D = standard error of the differences between means.

cottonseed pigment glands was only 1%. We believe that the difference in toxicity is due to some substance other than gossypol which either deteriorates or is altered on storage; since, of the two samples of pigment glands studied, the one showing lowered toxicity was from seeds which were processed about 6 months after the first sample was processed. The last columns of tables 2 and 3 show the average daily food consumption per rat. It will be observed that the difference in food consumption for rats on 0.256 and 0.512% cottonseed pigment glands (CPG) and for the controls was, on an average less than 2 gm/rat/day. This amount, we believe, is insufficient to cause the inhibition in growth observed.

In these studies no attempt was made to determine whether the addition to the diet of nutritive essentials such as vitamins, amino acids, or minerals has any measurable effect on the toxicity of the pigment gland constituent.

Hematological studies, consisting of red and white cell counts and hemoglobin estimation, were made on 4 occasions during the course of the feeding experiments. In all cases, no significant differences were observed among the rats on the various dietary levels of cottonseed meal and cottonseed pigment glands, and the controls.

Histopathological studies were made on tissues from all rats autopsied on completion of the chronic feeding experiments. There was no evidence of tissue damage that could be attributed to the supplements fed. The only finding which seemed to bear any relation to the diet was the presence of a finely granular, dark pigment in the Küpffer cells of the liver. Histochemical tests (Prussian blue reaction) for iron on this pigment were negative. This pigment was not found in the controls. The amount present did not seem to vary with the content of the supplements in the diet. In two rats on diets containing 0.512% of pigment glands, a mild suppression of spermatogenesis was noted.

Male and female rats on diets containing 0.256% of cottonseed pigment glands for approximately 100 days failed to reproduce. However, when the females were mated with males

that were on the basic diet alone up to the time of mating, all cast normal litters. By the 21st day only one litter of 6 survived. The females were repeatedly remated with the males on the 0.256% pigment gland diet, with no success. Keeping in mind the limited number of experiments, it would appear that pigment glands interfere with either spermatogenesis or the sexual urge and not with ovulation.

TABLE 4

Paired-feeding experiment; effect of cottonseed pigment glands (CPG) and gossypol (G) on body weight and food intake

(5 rats/group — 58 days)

TREATMENT	MEAN BODY WEIGHT			AVE. DAILY FOOD INTAKE
	At start [1]	At end [1]	Increase	
	gm	*gm*	*gm*	*gm* [1]
Control ad lib. ♂	49.2 ± 0.92	224.0 ± 6.47	174.8	13.2 ± 0.48
Control ad lib. ♀	46.5 ± 0.50	169.7 ± 3.1	123.2	11.4 ± 0.19
CPG 0.512% ♂	49.2 ± 1.07	113.2 ± 2.22	64.0	7.1 ± 0.10
Pair-fed controls	48.6 ± 0.81	143.4 ± 2.91	94.8	7.1
CPG 0.512% ♀	48.0 ± 0.64	103.0 ± 4.91	55.0	7.3 ± 0.32
Pair-fed controls	47.8 ± 0.59	127.0 ± 6.59	79.2	7.3
CPG 0.256% ♂	47.0 ± 0.84	164.2 ± 5.31	117.2	10.6 ± 0.53
Pair-fed controls	46.6 ± 0.92	190.4 ± 8.34	143.8	10.6
CPG 0.256% ♀	48.0 ± 0.53	144.6 ± 4.61	96.6	9.9 ± 0.33
Pair-fed controls	48.2 ± 0.50	159.6 ± 3.32	111.4	9.9
Gossypol 0.192% ♂	62.0 ± 0.89	115.8 ± 14.97	53.8	6.56 ± 0.28
Pair-fed controls	61.2 ± 0.74	155.6 ± 12.57	94.4	6.56
Gossypol 0.192% ♀	56.8 ± 1.60	99.2 ± 8.64	42.4	6.50 ± 0.44
Pair-fed controls	56.0 ± 1.52	122.8 ± 8.29	66.8	6.50

[1] Standard error of the mean.

Data on paired-feeding experiments with cottonseed pigment glands and gossypol are summarized in table 4. For comparison, data for rats having free access to the basic diet are also given. These results clearly indicate that the largest dietary level of cottonseed pigment glands, 0.512% for both male and female rats, elicited the greatest inhibition in growth and food consumption. The depressive effect on growth and appetite was more pronounced during the initial 15 days than during the remainder of the feeding period, which lasted

for 58 days. The growth rate of the pair-fed controls, limited in each case to the amount of food consumed by their respective mates fed cottonseed pigment glands, was definitely better, but not as great as that of the ad libitum controls. In both the male and female rats on the diet containing 0.256% of cottonseed pigment glands the inhibition in growth and food consumption was not as great as with rats on the higher concentration. There was, however, an inhibition in growth in cottonseed-pigment-gland-fed rats as compared with their respective mates. This inhibition indicates that most of the growth-depressing effect of cottonseed pigment glands cannot be accounted for by reduced food intake alone. Since no pathological changes in tissue were observed in rats on 0.512% CPG for approximately 185 days, it thus appears that cottonseed pigment glands exert not only an appetite-depressing effect but also a toxic action attributable to CPG per se. In all experiments the rats on the CPG diets grew slightly less than their litter-mate controls. In these experiments no record of water intake was kept.

In paired feeding studies with gossypol, a diet containing 0.192% gossypol caused an inhibition in growth and appetite of male and female rats comparable to that produced in rats on 0.512% cottonseed pigment glands (equivalent to 0.192% gossypol, table 4). The growth rate of the pair-fed control rats, limited to amounts of food consumed by individual rats on the gossypol diet, was definitely better than the growth rate of the latter. This indicates that the growth inhibition of the gossypol-fed rats cannot be accounted for on the basis of reduced food intake alone, but that gossypol played an important role in further depressing growth. The amount of gossypol ingested daily by the rats on the 0.192% gossypol diet was approximately 60 mg/kg/day over a period of 58 days without any fatalities. The rats were returned to the basic diet and after 30 days were reweighed. The control rats made a normal gain in weight, but the rats that had been on gossypol did not. The males appeared to be more affected than the females. These results are in agreement with those

of Eagle and Bialek ('50), where 50 mg/kg for 11 days resulted in depression in body weight below that of the controls.

Since the number of rats used in determining the acute oral toxicity of cottonseed pigment glands and gossypol was small, no attempt was made to determine the LD_{50}. However, from the data obtained it is clear that the acute oral toxicity of cottonseed pigment glands and gossypol is low.

Rats receiving cottonseed pigment glands in doses up to 3,000 mg/kg showed no evidence of toxicity within the first 24 hours after administration or during the next 30 days. In doses of 4,000 mg/kg, two died within 24 hours, three two to 7 days after administration, and one showed no ill effects. Gossypol, on the other hand, in doses up to 800 mg/kg did not appear to be toxic, while doses of 1,600 mg/kg were invariably fatal, although death was delayed 8 to 9 days. With a higher dosage (3,200 mg/kg), no deaths occurred within 24 hours, but all rats died within 6 days after administration. All rats receiving the fatal or near-fatal doses of glands or gossypol showed evidence of moderate to severe diarrhea. The degree did not appear to be proportional to the dose administered.

Rats receiving cottonseed pigment glands in three repeated doses of 2,000 mg/kg (equivalent to 730 mg gossypol) at 7-day intervals, or gossypol in doses of 500 mg/kg at 5-day intervals, showed no ill effects other than loss in body weight and anorexia. The loss in body weight, on an average, amounted to about 15% of the original weight and became apparent within 5 days after administration of either cottonseed pigment glands or gossypol. Food consumption during this period dropped from an average of 15 gm/rat/day to a low of approximately 3 gm/rat/day, and returned to normal in about 5 days. With cottonseed pigment glands, some of the rats developed diarrhea for a day or two after administration. With gossypol no evidence of diarrhea was observed in any of the rats. With lower dosages of cottonseed pigment glands, 1,000 mg/kg, or gossypol, 250 mg/kg, little or no loss of body weight or anorexia was observed. These

results are substantially in agreement with those reported by Eagle and Bialek ('50). Our studies, therefore, suggest that gossypol is the responsible agent in causing loss of body weight and anorexia. However, the diarrhea observed in rats given cottonseed pigment glands is in all probability due to some constituent, other than gossypol, present in the pigment glands.

SUMMARY AND CONCLUSIONS

The results of these experiments point to the conclusion that cottonseed meal free of pigment glands is devoid of any toxic principle as judged by the growth of rats and histopathological examination of visceral organs. Our results would indicate that hexane-extracted meal is not completely devoid of toxic principles or appetite-depressing factors, as judged by inhibition in the growth of rats on a diet containing 15% or more of the meal. This inhibition in growth cannot be accounted for completely by the slight difference in food consumed between rats on this diet and the controls or rats on a lower dietary intake of hexane-extracted meal. It would appear at first sight that the toxicity of hexane-extracted meal (15% level) may be due to the gossypol present in the meal, since 0.256% of pigment glands (equivalent to 0.096% gossypol) in the diet of rats produced an inhibition in growth comparable to that produced by the diet containing 15% of hexane-extracted meal. However, it is doubtful if gossypol is the chief responsible constituent, since rats on the 0.256% pigment gland diet processed from seeds stored for approximately 6 months were not similarly affected. This difference in toxicity of the two samples of pigment glands used may be accounted for on the basis of storage time before processing for removal of the pigment glands. The gossypol content of the two pigment-gland diets was extremely small, 0.096 and 0.0934%, respectively, as compared to 0.16% for the diet containing 15% of hexane-extracted meal. Comparable chronic toxicity studies of gossypol were not made, because of the unavailability of enough gossypol at the time the studies

with cottonseed meal and pigment glands were being carried out.

Paired feeding experiments with cottonseed pigment glands and gossypol would tend to confirm the above observations that decrease in growth is not entirely due to reduced food intake, but that gossypol plays an important role in depressing growth.

From the acute and subacute oral toxicological data obtained, it is concluded that the toxicity of cottonseed pigment glands and gossypol is low. Single oral doses of pigment glands of up to 3,000 mg/kg, or gossypol in doses of up to 800 mg/kg each, did not appear to produce any toxic symptoms.

The repeated administration of cottonseed pigment glands in doses of 2,000 mg/kg three times at 7-day intervals caused the rats to lose weight, depressed appetite, and produced diarrhea. Similarly, the repeated oral administration of gossypol in doses of 500 mg/kg at 5-day intervals had an effect similar to that of the pigment glands except that it did not produce diarrhea. Lower doses of pigment glands or gossypol were without effect.

The experimental data raise the question of whether or not chemical assay of cottonseed pigment glands for gossypol is a reliable index, since a difference has been observed in the toxicity of pigment glands processed at different times, while the gossypol content was approximately the same.

Since the completion of this work a paper by Lillie and Bird ('50) on chicks has appeared in which our findings on the toxicity of cottonseed pigment glands and gossypol are confirmed.

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THE AUGMENTATION OF THE PROVITAMIN A POTENCY OF CAROTENE WHEN FED IN MARGARINE [1]

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There are a number of reports in the literature which indicate that carotene under certain conditions may be less effective as a source of vitamin A than the preformed vitamin itself. These findings are undoubtedly to be ascribed to the fact that carotene is a hydrocarbon while vitamin A is an alcohol, and therefore a more polar substance. Although the variation in utilization of carotene and vitamin A may be almost entirely related to differences in absorption, the effects might conceivably be associated with the transformation of carotene to vitamin A.

The most important factor which aids in the absorption of carotene is the concomitant absorption of fat. Both rats and cats were shown by Ahmad ('31) to suffer a failure in the absorption and utilization of carotene on a low-fat diet, while the incorporation of 10% of fat in the food allowed an almost complete utilization of this carotenoid. Similar conclusions were reached by Coward ('36), Wilson et al. ('37) and van Eckelen and Pannevis ('38). Kemmerer and Fraps ('38)

[1] Paper 268 of the Department of Biochemistry and Nutrition, University of Southern California. These results were presented before the Southern California Section of the Institute of Food Technologists in a symposium on "Carotene in the Food Industry" on October 18, 1950.

noted an increased absorption of carotene by rats when the provitamin was fed in cottonseed oil,[2] while Shaw and Deuel ('44) observed a correlation between the amounts of fat and carotene absorbed by rats. Russell and co-workers ('42) confirmed these results on hens but likewise demonstrated that the absorption of vitamin A is not primarily affected by the level of fat in the diet.

The presence of non-absorbable hydrocarbons such as mineral oil has a greater deleterious effect on the absorption of carotene than on that of vitamin A. Rowntree ('31) first reported that the minimum quantities of vitamin A required for growth by rats was increased when mineral oil was added to the diet. It was found several years later that the absorption of carotene was effected to a much greater extent by mineral oil than was that of vitamin A (Dutcher et al., '34). That the absorption of carotene is lowered in man as well by the ingestion of mineral oil was demonstrated by Curtis and Kline ('39) by means of carotene plasma studies. These latter results were confirmed by Alexander and co-workers ('47).

The absence of emulsification in the intestine has a more pronounced effect on carotene utilization than on that of vitamin A. When emulsification is retarded by preventing bile from entering the upper digestive tract, as in choledochocolonostomized vitamin-A-deficient rats, orally administered carotene is ineffective in bringing about a cure of the deficiency, although the response to vitamin A remains positive (Greaves and Schmidt, '35a). When bile salts were administered in the diet to the animals which had been operated on, the rats were shown to be able to utilize carotene. As a further confirmation of this divergent behavior, Greaves and Schmidt ('35b) found that carotene failed to be utilized in jaundiced rats as well as in those with liver injury produced by phosphorus injection, although vitamin A could be utilized. The importance of bile in the absorption of carotene in dogs has been shown by Irvin, Kopala and Johnston ('41), making use of the loop technic.

[2] Wesson.

As a corollary to the above, the presence of emulsifying agents greatly augments the effectiveness of carotene, probably to a more pronounced degree than in the utilization of vitamin A itself. This fact was convincingly demonstrated by Adlersberg and Sobotka ('43) in man. Adlersberg and associates ('49) have recently reported that butter is a better vehicle for promoting carotene absorption than is cottonseed oil; however, when lecithin is added to cottonseed oil, the resulting carotene absorption is at a higher level than when the carotenoid is fed in butter. The improvement in absorption caused by lecithin may likewise occur in the case of vitamin A, even in normal individuals (Adlersberg et al., '48; Kern et al., '49). The absorption of high-melting fats is also facilitated by the addition of lecithin (Augur et al., '47).

That anti-oxidants play an important role in augmenting the biological response following the feeding of critical amounts of vitamin A, and especially carotene, has been amply demonstrated (Hickman and Harris, '46). In order to control this variable in biological assays, it has become common practice to supplement with tocopherols the limpid vegetable oil, namely cottonseed oil, used in diluting the vitamin A supplements. The usual range of tocopherol concentration in the fortified diluent oil is 0.3 to 0.5%.

While it is known that the transformation of carotene to vitamin A occurs in the wall of the intestine in the rat (Mattson et al., '47; Thompson et al., '47; Glover et al., '47), chicken (Cheng and Deuel, '50) rabbit, sheep, goat (Goodwin and Gregory, '48; Goodwin et al., '46) and pig (Thompson et al., '47), there is considerable evidence that it does not necessarily take place at this site in the case of the dog, cow and man. However, there are no experimental findings which demonstrate alterations in the mechanism for bringing about the conversion of carotene to vitamin A which do not involve changes in absorption from the intestine. This applies both to the species in which carotene is changed to vitamin A in the intestinal wall and to those where the transformation may occur in another site.

In view of the foregoing observations, margarine would seem to be an especially effective vehicle for permitting a high utilization of carotene. It furnishes (a) a readily-digestible fatty medium for dissolving the carotene, (b) emulsifying agents known to aid in the absorption of carotene, and (c) a vehicle in which carotene should exhibit considerable stability. Most margarines include appreciable proportions of lecithin, monoglycerides and diglycerides, which are permitted as optional ingredients in the standard for the product (Federal Security Agency, '49). The first of these emulsifying agents eliminates spattering when foods are fried with margarine, while the mono- and diglycerides prevent separation into aqueous and fat phases during the life of the product. Hydrogenation of the vegetable oils used in making margarine of course increases the stability of the oils against oxidative rancidity. In addition, margarines made of vegetable oils [3] retain a major portion of natural anti-oxidants, the tocopherols, originally present in the limpid oil (Harris et al., '50). The supplementation of the refined, hydrogenated, deodorized oils with lecithin eliminates in large measure the pro-oxidant effects of trace metals found in all fats and oils (Dutton et al., '49). Hence, one would also expect carotene (and vitamin A) to be more resistant to oxidation when dissolved in a stabilized fat.

In the present study data have been obtained demonstrating that there is indeed an augmentation of the provitamin A potency of carotene when fed in margarine. A plausible explanation for this effect is suggested by the results of the *in vitro* tests reported. Since carotene, while supplying a part of the vitamin A potency of margarine, serves also to color the product, its use as the coloring agent in yellow margarine is in the interest of good nutrition.

[3] About 97.5% of all margarines currently manufactured in the United States are made exclusively of vegetable oils (U.S. Dept. Agr., '50).

EXPERIMENTAL

All margarine samples used in the present study were, with one exception, from materials manufactured as in commercial production. One margarine sample was made in the pilot plant; it contained carotene as the sole source of vitamin A, but at the same level found in the commercial yellow product. Of necessity, the investigation was limited to the product made by one manufacturer, since of all margarines made in the United States, it alone is colored with carotene.[4] However, since the composition of margarine is defined within narrow limits (Federal Security Agency, '49), the results reported in this paper should be applicable to all margarines made exclusively with hydrogenated vegetable oils.

The carotene concentrate [5] used to color the margarine and contribute a portion of the vitamin A potency was a 30% suspension (by weight) of 90% beta-carotene and 10% alpha-carotene in cottonseed oil. The concentrates of preformed vitamin A were either a blend of food fish liver oils of high potency [6] or a solution of distilled vitamin A esters.[7]

Multiple level (three) biological assays were conducted on the more important test samples, using an elaboration of the procedure described in the U. S. Pharmacopoeia XIV. The U.S.P. procedure, as published, calls for a single level assay to determine whether or not a given product satisfies the label claim. In order to permit estimates of potency to be calculated from the animal data, it is necessary to feed both test and reference oils at two or more levels. In addition to multiple level assays, a relatively large number of single level assays were conducted on margarines purchased in the open market. The present report includes data on biopotency ob-

[4] Yellow Nucoa margarine.

[5] Barnett's microcrystalline carotene, obtained from the Barnett Laboratories, Long Beach, California.

[6] Obtained from Halibut Liver Oil Producers, Seattle, Wash.

[7] Obtained from Distillation Products, Inc., Rochester, N.Y.

tained independently by the University of Southern California and by two commercial laboratories.[8]

Carotene was determined by spectrophotometric measurement of the light absorption of a cyclohexane solution of the test materials. The extinction coefficient values ($E\frac{1\%}{1\text{ cm}}$ 455 mμ) were multiplied by 660 to convert to U.S.P. units of vitamin A.[9] In assays of the fortified margarines, the Beckman spectrophotometer was set at 100% transmission, with the oil separated from a blank sample of margarine (similar to the fortified sample in all ways except lacking the carotene or the preformed vitamin A concentrate or both) and dissolved in cyclohexane.

There is no evidence of any appreciable isomerization of the all-*trans*-β carotene in the fresh or stored margarine. Carotene is known to be especially stable in oil solution. Concentrated carotene samples in oil from the same source [10], freshly prepared and after standing one year, were both shown by Professor L. Zechmeister to contain less than 1% of the neo-β-forms. For this reason the use of the wave length of 455 mμ in cyclohexane for the analysis would appear to be justified.

In assays of the preformed vitamin A concentrates, the spectrophotometric and colorimetric ($SbCl_3$) methods described in U.S.P. XIV were employed. The margarine samples were assayed spectrophotometrically, using the appropiate blank sample as described above in testing for carotene. Small corrections were applied to the $E\frac{1\%}{1\text{ cm}}$ 328 mμ values for light absorption at this wave length due to carotene, when present,

[8] The authors are indebted to Dr. Bernard L. Oser of the Food Research Laboratories, Long Island City, N.Y., and to Dr. George K. Falk of the Laboratory of Industrial Hygiene, New York, N.Y., for the data furnished.

[9] Pure beta-carotene, kindly supplied by Dr. L. Zechmeister, California Institute of Technology, Pasadena, was found to have an $E\frac{1\%}{1\text{ cm}}$ 455 mμ value in cyclohexane of 2,400. On the basis that 0.6 μg of pure beta-carotene furnishes 1.0 U.S.P unit of vitamin A, the factor for converting $E\frac{1\%}{1\text{ cm}}$ 455 mμ values into U.S.P. units of vitamin A is 694. The factor, 660, used in the current study takes into consideration that 10% of the carotene in the preparations employed was the alpha-form, and that this exhibits only about one-half the potency of beta-carotene.

[10] See footnote 5, page 375.

and to the participation of carotene in the $SbCl_3$ tests. The details of the physico-chemical methods employed are presented in a paper in the process of publication (Luckmann et al., '51).

Tests of the resistance of certain test oils to oxidative deterioration were made by the active oxygen method (Swift Stability Test), using the procedure described by Riemenschneider et al. ('43) and based upon the original article by King and associates ('33). In addition, the stability of carotene (and preformed vitamin A) in these oils was evaluated, using an accelerated holding test for this purpose.

Measurements of emulsifying potentialities were based upon determinations of the interfacial tension of the test oils stratified over water [11] (du Nouy, '25).

RESULTS AND DISCUSSIONS

A summary of the non-biological and of the multiple level bioassays of both the vitamin A concentrates and the fortified margarine samples is presented in table 1.

When vitamin A or carotene is fed dissolved in cottonseed oil (containing up to 0.5% added tocopherols), the agreement between the spectrophotometric (whole oil) estimates of the vitamin A potency of the concentrates and the average results of bioassay from several laboratories is quite good.[12] Under such conditions the ratios of potency by biological assay to those by spectrophotometric determination vary between 0.95 and 0.98. The results are uniform irrespective of whether distilled vitamin A esters, a blend of food fish liver

[11] A Cenco-du Nouy tensiometer, Model No. 70540, was used. The details of the test procedure are given in Bulletin 101 (pp. 11–12) furnished by the manufacturer, Central Scientific Company, Chicago, Ill.

[12] Whereas U.S.P. specifications permit a ratio of 1.30 for colorimetric ($SbCl_3$) to corrected spectrophotometric estimates, concentrates of preformed vitamin A of high quality used to fortify margarine have a ratio of about 1.15. It has been found as a result of an extensive collaborative study conducted on such oils (Melnick et al., '51) that the spectrophotometric estimate based upon analysis of the whole oil sample is a reliable estimate of the true biological potency.

TABLE 1

The comparative biological and non-biological assays for vitamin A and carotene in concentrates and in margarines

SAMPLE	NON-BIOLOGICAL ASSAYS				MULTIPLE LEVEL BIOASSAYS [1]		
	Whole oil	Unsaponifiable extract					
	Spectrophotometric (1)	Corrected spectrophotometric U.S.P. XIV (2)	Colorimetric ($SbCl_3$) U.S.P. XIV (3)	Ratio of (3) to (2)	Value found	Average (4)	Ratio of (4) to (1)
In concentrates		*U.S.P. units/gm*				*U.S.P. units/gm*	
Distilled vitamin A esters	204,600	189,800	209,100	1.10	209,000(A) 197,000(B) 183,000(C)	196,000	0.96
Blend of food fish liver oils of high potency	199,400	175,100	201,100	1.15	203,000(A) 171,000(B) 197,000(C)	190,000	0.95
Carotene concentrate	386,400	.....	.....	...	377,000(B)	377,000	0.98
In margarines		*U.S.P. units/lb.*				*U.S.P. units/lb.*	
Distilled vitamin A esters	18,300	.....	19,000	...	18,500(A) 15,400(B)	17,000	0.93
Blend of food fish liver oils of high potency	17,000	.....	17,600	...	19,300(A) 17,300(B)	18,300	1.08
Carotene	5,850	.....	.....	...	7,100(A) 7,900(B)	7,500	1.28
Above sample stored 7 weeks at room temperature	5,100	.....	.....	...	6,200(B)	6,200	1.21
Carotene concentrate + distilled vitamin A esters	17,400	.....	18,100 [2]	...	21,900(A) 18,200(B)	20,100	1.16
Above sample stored 7 weeks at room temperature	15,200	.....	17,600 [2]	...	19,000(B)	19,000	1.25

[1] The letters in parentheses refer to the laboratories participating in the biological assays. Each laboratory conducted multiple level assays on the products indicated, feeding the test material at three assay levels and the U.S.P. Reference Oil at two levels.

[2] Total vitamin A content based upon colorimetric ($SbCl_3$) test for preformed vitamin A plus that derived from spectrophotometric analysis for carotene content.

oils of high potency, or carotene concentrates are used as the source of vitamin A.

Essentially the same results are obtained when preformed vitamin A is fed as a constituent of margarine. Thus the ratio of biological to non-biological estimate of potency was 0.93 for the distilled vitamin A esters and 1.08 for a natural vitamin A product (blend of food fish liver oils of high potency). On the other hand, the biological response to carotene when fed in margarine either alone or with vitamin A is considerably enhanced over what one would expect on the basis of spectrophotometric assay. The ratios of biological to non-biological estimates under such conditions were 1.28 and 1.21, respectively, for a margarine sample containing carotene alone before and after storage at room temperature for a period of 7 weeks. The corresponding ratios for a product containing both carotene and vitamin A were 1.16 and 1.25 respectively, before and after a similar storage period.

The greater-than-theoretical response following the feeding of carotene in a margarine vehicle to vitamin A-depleted rats is confirmed by the data presented in table 2. In this series the results obtained in single level biological assays of the last 18 samples of margarine purchased in the open market are compared with the results yielded by non-biological tests.

According to assays conducted on the concentrates of preformed vitamin A and the quantities used to fortify each batch of margarine, the white product should have contained 18,000 U.S.P. units of vitamin A per pound. Numerous routine spectrophotometric tests versus the proper blank controls have indicated that there is, however, a processing loss of about 5% (900 U.S.P. units per pound) of the vitamin A in manufacturing the product, and a negligible loss of about 2% when the product is held for an extended period of three to 4 months at 45°F. Thus, the product as picked up in the open market actually contains about 17,000 U.S.P. units per pound. The yellow margarine is likewise fortified to the 18,000 U.S.P. units per pound level, 6,000 to 7,000 U.S.P. units being derived from the added carotene and the remainder from preformed vitamin

TABLE 2

Routine vitamin A assays of open-market samples of margarine

WHITE MARGARINE					YELLOW MARGARINE				
Sample no.	Colorimetric ($SbCl_3$) value	Single level bioassays: Gain in body weight			Sample no.	Colorimetric ($SbCl_3$) + spectrophotometric value	Single level bioassays: Gain in body weight		
		Assay group	Reference group	Ratio			Assay group	Reference group	Ratio
	U.S.P. u./lb.					*U.S.P. u./lb.*			
1	18,100	39.4	38.9	1.01	10	19,800	45.4	34.1	1.33
2	18,800	47.3	40.3	1.17	11	18,000	43.6	36.6	1.19
3	19,200	53.5	46.0	1.16	12	19,100	49.3	36.3	1.36
4	16,700	42.1	40.9	1.03	13	18,200	51.5	49.9	1.03
5	18,800	43.8	41.5	1.06	14	18,300	54.5	44.9	1.21
6	18,900	48.0	42.6	1.13	15	18,700	48.8	39.5	1.24
7	19,200	47.3	38.7	1.22	16	17,000	46.5	40.9	1.14
8	19,000	41.3	39.6	1.04	17	17,300	53.8	40.9	1.31
9	18,100	53.4	39.5	1.35	18	17,400	55.5	40.9	1.36
Average	18,500	46.2	40.9	1.13	Average	18,200	49.9	40.4	1.24
Estimate of biological potency [1] 17,100 U.S.P. units/lb.					Estimate of biological potency [1] 19,200 U.S.P. units/lb.				

[1] See text.

A. The yellow product, according to routine control non-biological assays, also contains about 17,000 U.S.P. units per pound, since the same processing losses prevail. The average values of 18,500 U.S.P. units, obtained by colorimetric ($SbCl_3$) assay of the unsaponifiable extract of the white margarines, and 18,200 units in the case of the yellow margarines, are known to be over-estimates (about 7.5%) of the true vitamin A content (Luckmann et al., '51).[13]

The bioassays reported in table 2 were all single level U.S.P. tests, the products being assayed at the potency level declared on the carton, i.e., at 15,000 U.S.P. units of vitamin A per pound. A passing assay is one in which the average net gain in body weight of the depleted assay rats during the 28-day test period equals or exceeds that of the standard reference group during the same period. It will be apparent that in every case the growth response of the animals receiving the vitamin A supplement in margarine oil was greater than that of the rats receiving the U.S.P. Reference Oil in cottonseed oil with added tocopherols. This was anticipated because of the appreciable vitamin A overage in fortifying the margarine. However, the performance of the animals receiving the carotene-supplemented margarine was, relative to that of the animals dosed with the U.S.P. Reference Oil, markedly superior, exceeding that anticipated on the basis of the values obtained by non-biological assays. Thus, whereas the ratio in gain in body weight of the animals receiving the white margarines to that of the rats receiving the U.S.P. Reference Oil was 1.13 (standard error of $\pm$ 0.034), the ratio in the biological assays of the yellow margarine was 1.24 (standard error of $\pm$ 0.037). This difference, "t" = 2.12, is statistically significant at the probability level of $p = 0.05$. There was no significant difference in vitamin A content between white and yellow margarines when assayed by the non-biological procedures. In other words,

[13] It was not possible to test these margarine samples by the more accurate (specific) spectrophotometric procedure, since control unfortified margarine oil samples corresponding to the fortified products were not available to permit correction for irrelevant light absorption.

carotene in margarine gives a greater-than-theoretical vitamin A response.

By interpolating the average growth responses of the assay animals in the two series on the average log-dose response curve obtained with the U.S.P. Reference Oil in the course of the multiple level biological assays previously reported, it was possible to obtain an estimate of the biological potency of the two types of margarine [14]. For the white margarines, an average value of 17,100 U.S.P. units of vitamin A per pound was obtained, in close agreement with the values regularly obtained in spectrophotometric control tests of the margarine product; whereas for the yellow margarine the average value was 19,200 U.S.P. units, or 12% greater. Considering that only 35 to 40% of the total vitamin A in the yellow margarine samples was furnished by carotene (the remainder being supplied by preformed vitamin A), the augmentation of biological response attributable to the administration of carotene in margarine was, in this particular series, from 30 to 35%.

In an attempt to elucidate the possible mechanism for the greater-than-theoretical availability of carotene when fed in a margarine vehicle, a number of *in vitro* tests were conducted. These were concerned with: (a) an evaluation of the relative stability of carotene and preformed vitamin A when dissolved in each of the diluent feeding oils—margarine oil plus lecithin and mono- and diglycerides in one case, and the limpid cottonseed oil with added tocopherols in the other; and (b) an evaluation of the ease of dispersing the diluent feeding oils in water. In the introduction to this paper reports were cited in support of the hypothesis that vitamin A, preformed and particularly as the provitamin, exhibits greater physiological availability when fed along with emulsifying and anti-oxidant agents.

The results of the *in vitro* tests are summarized in tables 3 and 4. It will be noted from the data presented in table 3 that

[14] The dose-response relationships of the reference animals in the multiple-level assays of the margarine samples (see table 1) were quite uniform. Thus, for an 80% increase in dosage level, namely, 1.25 U.S.P. units to 2.25 U.S.P. units per day, the animals receiving the higher dose gained 50, 60, 70, 50, 65, or 60% (average 60%) more in body weight than those on the lower dose of vitamin A.

the margarine oil, a stabilized blend of hydrogenated vegetable oils, was far more resistant to oxidative deterioration than the limpid cottonseed oil. Whereas this particular cottonseed oil had an active oxygen method stability value of only 4 hours, the margarine oil had a stability value of 118 hours. The retention of preformed vitamin A and carotene in the marga-

TABLE 3

Stability of diluent feeding oils and of preformed vitamin A and carotene in these oils

TEST	DILUENT OIL	RESULTS	
Active oxygen method stability test (peroxide value of 100 as the end-point)	Limpid cottonseed oil + 0.5% tocopherols	4 hours	
	Margarine oil plus additives (lecithin and mono- and diglycerides)	118 hours	
Stability of preformed vitamin A in diluent oil [1]	*Limpid cottonseed oil plus tocopherols, fortified:*	*Vitamin A retained U.S.P. units/gm*	*%*
	Freshly prepared	45.2	100
	After 88 hrs. at 140°F.	33.0	73
	After 112 hrs. at 140°F.	29.3	35
	Margarine oil plus additives, fortified:		
	Freshly prepared	43.0	100
	After 88 hrs. at 140°F.	41.5	96
	After 112 hrs. at 140°F.	41.5	96
Stability of carotene in diluent oil [1]	*Limpid cottonseed oil plus tocopherols, fortified:*		
	Freshly prepared	17.5	100
	After 88 hrs. at 140°F.	13.8	79
	After 112 hrs. at 140°F.	12.8	73
	Margarine oil plus additives, fortified:		
	Freshly prepared	17.7	100
	After 88 hrs. at 140°F.	15.9	90
	After 112 hrs. at 140°F.	15.7	89

[1] The diluent oils were fortified with preformed vitamin A (distilled vitamin A esters) or with the carotene concentrate (90% beta-, 10% alpha-carotene) to levels approaching those found in fortified margarine oils. Twenty-gram portions of each fortified oil were placed in 100-ml open beakers and stored in the dark at 140°F. The inside diameter of the beaker was 5 cm and the oil depth was 1.5 cm.

rine oil was definitely superior to that in the cottonseed salad oil.

Measurements at 37°C. of interfacial tension, the diluent oils stratified over water, reflect the ease with which these oils can be dispersed in water at body temperature. The values listed in table 4 demonstrate that the margarine oil containing the added emulsifying agents was far more easily dispersible. Whereas the interface between the cottonseed oil and water was well rounded and sharp throughout the period of test, the

TABLE 4

Interfacial tension measurements[1] of the diluent feeding oils at 37°C. stratified over water at 37°C.

CONTACT TIME BETWEEN OIL AND WATER	INTERFACIAL TENSION VALUES	
	Limpid cottonseed oil + 0.5% tocopherols over water	Margarine oil plus additives (lecithin and mono- and diglycerides) over water
hours	*dynes per cm*	*dynes per cm*
0 (1 min.)	22.6	2.2
0.5	22.3	1.9
1.0	22.2	1.8
18	17.3	1.3
19	17.2	1.5

[1] Fifteen grams of oil were floated on 50 gm of distilled water in a glass dish 6.0 cm in diameter and 3.5 cm in height. A Cenco-du Nouy interfacial tensiometer was used for the measurements.

interface between the margarine oil and water became horizontal and noticeably hazy in 15 to 20 minutes after contact, and remained so thereafter.

Whereas preformed vitamin A as well as carotene was subjected to the same environmental factors promoting greater physiological availability, only in the case of carotene was increased availability detected. In the studies cited earlier in this paper, it was pointed out that carotene responds to a greater degree to the favorable effects resulting from the concomitant ingestion of emulsifying and anti-oxidant materials.

Apparently the latter are present in too small concentrations for augmenting the biological response following the ingestion of preformed vitamin A in margarine. The finding that carotene in margarine exhibits physiological availability in excess of 100% is not unusual. Factors capable of stabilizing a labile nutrient in the gastrointestinal tract or improving absorption, or both, have been shown to promote a greater-than-theoretical biological response (Melnick and Oser, '47). Vitamin content and vitamin availability are not necessarily synonymous.

Brief mention should be made of the remarkable stability of vitamin A (preformed or as the provitamin) in margarine. When held for an extended period of 7 weeks at room temperature, a loss of only 11% occurred according to the biological assays, and a loss of only 13% according to the spectrophotometric assays. When properly stored, i.e., at 45°F., the loss is only about 2%, even after three to 4 months of storage.

SUMMARY

Preformed vitamin A, derived from an oil solution of distilled vitamin A esters or from a blend of food fish liver oils of high potency, promotes a growth response of the same order of magnitude when fed in either cottonseed oil or in margarine to vitamin A-depleted rats. On the other hand, carotene when administered in margarine is responsible for a greater-than-theoretical response. In the margarine vehicle the physiological availability of vitamin A from carotene is about 130%, i.e., 30% greater than when fed in limpid cottonseed oil, despite supplementation of the latter with tocopherols.

In vitro tests have demonstrated that both preformed vitamin A and carotene are more stable in the hydrogenated and anti-oxidant-supplemented blend of vegetable oils constituting margarine oil, and that the presence of the added emulsifying agents, lecithin and mono- and diglycerides, increases the dispersibility at body temperature of margarine oil in water. The observation that only carotene responds to the factors in margarine which promote greater physiological availability

is attributed to carotene's being more sensitive than preformed vitamin A to the effect of environmental factors capable of augmenting the vitamin A response.

Both vitamin A and carotene are relatively stable in margarine, even when held at room temperature over a prolonged period.

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THE DEMONSTRATION OF A COPPER DEFICIENCY IN YOUNG GROWING PIGS[1, 2]

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FIVE FIGURES

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Elvehjem and Hart ('32) demonstrated that copper together with iron is necessary for hemoglobin formation in the pig. Schultze, Elvehjem and Hart ('36b) suggested that a blood copper level as low as 20 μg per 100 ml is sufficient for a slow formation of hemoglobin in the pig, but that hematopoiesis ceases if the level falls below 10 μg per 100 ml of blood in the young pig. Although the level of copper required by a young growing pig was not established, these workers reported that 2 to 4 mg per pig per day were sufficient for hemoglobin formation during recovery from anemia produced on a whole milk diet supplemented with iron and manganese.

This paper is a preliminary report of experiments to determine the copper requirements of young growing pigs. During the progress of these experiments, pigs fed a whole milk diet low in copper developed deficiency symptoms which, to our knowledge, have not been reported previously.

EXPERIMENTAL

Three experiments were conducted. The test animals were Chester White pigs farrowed indoors on a concrete floor. In the first two experiments each pig received, immediately after

[1] This work was supported by a grant recommended by Battelle Memorial Institute of Columbus, Ohio, and made by the Kennecott Copper Corporation.

[2] Hormel Institute publication 53.

birth, an iron pill containing 290 mg of reduced iron. The pigs in the third experiment did not receive an iron pill. All pigs were taken from their dams at 4 to 10 days of age and placed in aluminum cages for the duration of the experimental periods. Each cage was equipped with a stainless steel feed trough and a false bottom of cadmium plated expanded metal screen. Raw whole cow's milk, to which were added 1.05 mg of manganese and 0.21 mg of iodine per liter, served as the basal diet. The milk was collected in a stainless steel milking vessel and then transferred into glass jars for cooling and transit. When washed, the glass jars and feed pans were thoroughly rinsed with copper-free double distilled water. Periodic analyses of the basal diet showed it to contain from 0.05 to 0.15 mg of copper per liter, with a mean of about 0.08 mg per liter. Once weekly amounts that would be at least 58 I.U. of vitamin A and 5.6 I.U. of vitamin D [3] per kilogram of body weight per day were given orally to each pig. When from 4 to 5 weeks of age, the pigs in the second and third experiments received a commercial tissue vaccine [4] as immunization against cholera.

Blood hemoglobin values were obtained on each animal weekly by the acid hematin procedure, employing a Fisher electro-hemometer. Beginning the 5th week of the second experiment and through the entire third experiment, blood copper analyses were made on each animal every two weeks. The carbamate micromethod of Coulson ('37) was used for the milk and blood copper determinations. The pigs were weighed weekly and when they had become adjusted to the dietary regime, the daily milk consumption of each animal was recorded.

RESULTS

Experiment I

Seven 10-day old pigs were penned individually and fed whole milk ad libitum. All animals were drinking milk within

[3] Mead's Oleum Percomorphum, 60,000 units of vitamin A and 8,500 units of vitamin D per gram.

[4] Boynton's.

24 hours. Ten milligrams of iron as a ferric sulfate solution were fed to each pig daily until it was 30 days of age. At this time the supplemental iron was discontinued for three animals, and was increased to 15 mg per day for the remainder.

Because of prolonged unthriftiness, one pig was killed 30 days after the start of the experiment. One other animal died of a coronary condition on the 53rd day.

Figure 1 gives a graphic representation of blood hemoglobin values for two representative animals. After an initial rise there was a gradual decrease for all animals. When iron was omitted from the diet on the 30th day, the decrease in hemoglobin was not accelerated. However, a somewhat lower level was eventually obtained for these animals than for those that continued to receive iron. Increasing the level of iron from 10 to 15 mg per pig per day for the remaining animals had no effect on the gradual decrease of hemoglobin values. The feeding of 2 mg of copper per day in the absence of iron (pig 2, fig. 1) did not stimulate the regeneration of hemoglobin. Increased hemoglobin formation was initiated only when both iron and copper were added to the diet.

During the 4th week an unusual leg condition was observed in one animal. The front legs were spread at the top as though the body attachment had weakened, while the lower portion of the leg turned in, exhibiting a somewhat bowed appearance. The hind legs were abnormally crooked and lacked rigidity. Some of the leg joints were slightly swollen and reddened. These symptoms, with some variation, gradually appeared in all of the animals (plate 1). Eventually the hind legs were drawn under and, although paralysis did not appear, the ability to use the legs as a means of body support was lost. Some animals assumed a sitting position for a period before also losing the use of the front legs. By the 10th week, 4 pigs were unable to stand.

Outwardly the symptoms resembled rickets. However, doubling and in some cases trebling the vitamin A and D intake had no therapeutic effect. Iron as a supplement in the absence of copper did not prevent the further development of symp-

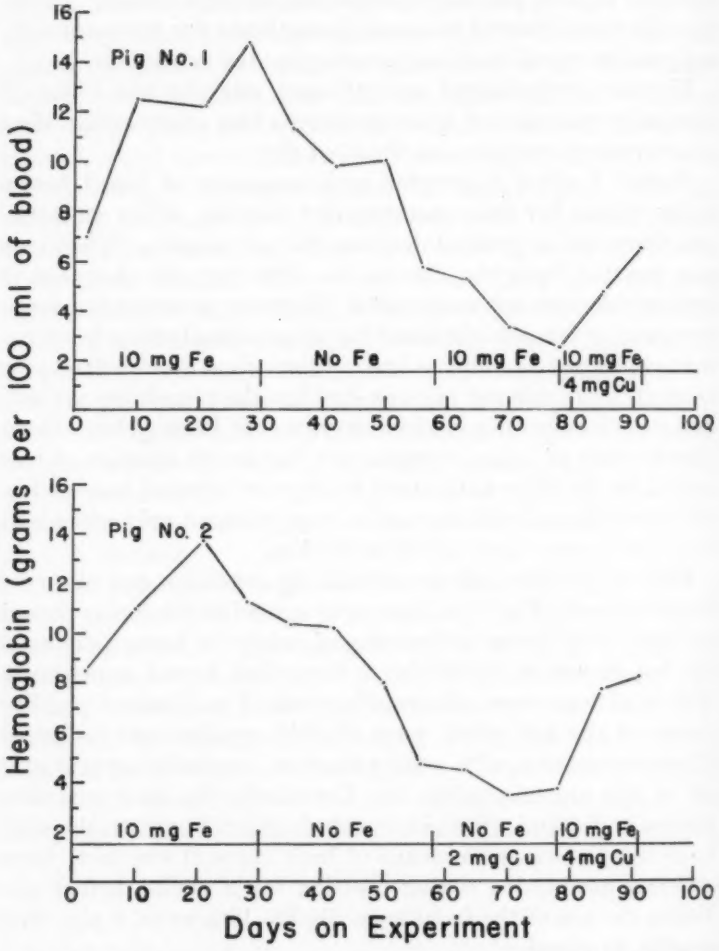

Fig. 1 A graphic representation of the blood hemoglobin values for pigs fed a low iron and copper diet and the subsequent response to the addition of these minerals to the diet.

toms. Supplementation with 2 mg of copper per day in the absence of iron appeared to arrest the further development of the condition but did not have a curative effect on the existing symptoms. In all animals where copper was fed in the presence of iron, marked improvement was demonstrated. The animals that were unable to stand could rise, and different degrees of leg straightening occurred after 10 to 14 days of supplementation. The reversibility of the visible portion of the syndrome varied among animals, and in only one case did it progress to apparently complete recovery. One animal received iron but no copper throughout the experiment. By the 10th week this pig was unable to stand; it progressed to an extreme deficiency state and died on the 94th day. The experiment was terminated on the 98th day.

Experiment II

Fifteen pigs representing two litters were taken from their dams when 4 and 5 days of age. They were placed in the metal cages and allowed 4 days in which to become adjusted to the basal diet before the start of the experiment. The three pigs serving as positive controls were fed the basal diet plus 10 mg of iron and 2 mg of copper per pig per day. The remaining 12 pigs were fed the basal diet plus 10 mg of iron per pig per day.

Nine of the 12 pigs on the low copper diet died before the termination of the experiment. Two died the first week from undetermined causes, and 4 died between the 30th and 35th days of the experiment. Of the latter animals, two died of bronchial pneumonia and two from shock following cholera vaccination. Deaths of the other three occurred during the 7th week of the experiment. At the time of death, these three pigs were anemic and possessed moderate to severe deficiency symptoms as described under experiment I.

The animals on the low copper diet developed a progression of symptoms which paralleled those produced in experiment I. Crookedness of the forelegs was noted as early as the 4th week. By the 6th week, the symptoms were moderate to severe

in all the deficient animals. The control animals which were receiving 2 mg of copper per day at no time showed leg crookedness or lack of muscular coordination. Visible symptoms of a deficiency generally appeared when the blood hemoglobin level was from 4 to 6 gm per 100 ml of blood. The front legs became weakened in the region of the carpal joints and turned in or out. In a few cases, prior to the appearance of this syndrome, the area immediately above the hoof and surrounding the joints became reddened. Standing then appeared to be painful; however, this persisted for only a short time. In addition to the crookedness, the front legs became spread as though the body attachment had weakened. The hind legs were excessively flexed at the hocks and had a turning or pivot action when the animal was standing. This condition progressed until the feet were so far forward that the animal could not stand. In general, the animal showed some loss of vitality, which seemed to be correlated more closely with the progression of the anemia than with the leg condition. Until the symptoms were severe, the rate of growth of the deficient animals closely paralleled that of the control animals. Shortly before death, some pigs drank poorly but otherwise the appetite did not seem markedly impaired.

By the 6th week the blood hemoglobin values for the copper deficient animals were 4.9, 4.1, 3.4, 3.3, 4.3 and 4.4 gm per 100 ml of blood, and values for the controls were 7.7, 8.5, and 7.9.

The blood copper levels of the three control pigs, after 7 weeks, were 175, 157, and 121 mg per 100 ml, as compared to 34, 29, and 24 for the deficient animals. In all instances the blood copper level of deficient animals showed a slow but gradual increase after copper supplementation.

During the 7th week of the experiment the fore and hind legs of three deficient animals and of one control animal were x-rayed. The roentgenograms showed no evidence of rickets in the deficient animals, but did bear out the malalignment of leg bones which was visibly apparent.

Samples of leg muscle tissue from deficient animals that died during the 7th week were examined histologically by Dr. W. A. Bennett of the Mayo Clinic, Rochester, Minnesota.[5] Preliminary findings indicated a marked atrophy and degeneration of the muscle fibers, which was associated with interstitial edema. Very little inflammatory reaction appeared except an occasional blood vessel surrounded by lymphocytes.

A program of copper supplementation was begun on the three remaining deficient animals at the end of the 7th week. One pig was fed 0.5 mg per day, and the other two, 1.0 mg per day. The pig fed 0.5 mg showed immediate improvement, and progressed from a condition of moderate crookedness of the fore and hind legs to a normal appearance in three weeks. One of the animals fed 1.0 mg of copper per day showed slow improvement, which continued until it was killed for tissue analyses 8 weeks later. The other pig showed no improvement after 13 days, at which time 4.0 mg of copper were given as a supplement per day. No visible change was brought about by this higher level of copper over a period of 5 weeks. At this time the animal was killed for autopsy.

Experiment III

From data collected in the first two experiments, it was noted that the deficiency symptoms appeared when the copper intake was about 0.035 mg per pound of body weight per day. In this experiment, the intake of copper relative to body weight was controlled in an attempt to establish a critical level or requirement for the pig under these experimental conditions.

Twenty pigs, 5 and 6 days old, from two litters were isolated and, after a 4-day adjustment period, were divided into 6 lots. Lot 1 consisted of 5 animals that served as negative controls and were fed the basal diet plus 20 mg of iron per day. This lot received only the copper present in the

[5] Dr. W. A. Bennett and Dr. G. M. Higgins of the Mayo Clinic, Rochester, Minnesota, were consulted with regard to the pathological condition involved. Their interest and subsequent work with us on this problem are greatly appreciated.

milk. Lots 2, 3, 4 and 5 each consisted of three animals. Each pig received the basal diet plus 20 mg of iron per day and the lots were kept on the following copper intakes: lot 2, 0.025; lot 3, 0.035; lot 4, 0.050 and lot 5, 0.70 mg per pound of body weight per day. These levels were maintained by feeding supplementary copper in addition to that present in the milk consumed. The intake of copper relative to body weight was calculated for each animal at least once a week. The 6th lot comprised three animals that were fed the milk diet plus 2 mg of copper per day but no additional iron. The blood hemoglobin level of each animal was determined weekly and blood copper values were obtained every two weeks.

The three animals in lot 6, in contrast to the pigs fed iron, showed an immediate and rapid decline in blood hemoglobin. The values for these animals at 4 weeks were 2.3, 3.3, and 3.8 gm per 100 ml of blood, at which time the blood copper levels were 222, 253, and 227 mg per 100 ml, respectively. The blood copper levels remained high throughout the experiment and seemed unaffected by the deficiency of iron. The pigs remained small and became very rough in appearance. One died on the 40th experimental day and the remaining two were killed on the following day for tissue analyses. These iron-deficient animals at no time showed any of the outward symptoms noted for the copper-deficient animals.

Deficiency symptoms were apparent in lots 1 and 2 within 4 weeks and appeared in two of the pigs in lot 3 by the 6th week. The milk consumption of the animals in lot 1 was sufficient to allow a copper intake during the first 4 weeks which closely approximated the intake of the animals in lot 2. Therefore the symptoms appeared in the two lots simultaneously. By the 6th week these 6 animals were markedly deficient.

During the 7th week it was decided to sacrifice all animals for histological examination. The microscopic study of many of the body tissues is now being conducted, the results of which will be reported at a later date. When sacrificed, the mean blood copper values for the animals of lots 1, 2, 3, 4 and 5 were 44, 40, 63, 92 and 145 mg per 100 ml of blood.

DISCUSSION

Except for the anemia, the deficiency symptoms herein described in young pigs receiving only the copper present in raw whole cows' milk were not observed by Elvehjem and Hart ('32) or by Schultze, Elvehjem and Hart ('36 a,b) in their experiments with pigs fed a whole milk diet supplemented with iron.[6] It is possible that the different experimental environments provide the reason for the different responses of the animals. Such factors as the source and handling of the milk and the care and housing of the young animals may play an important role in a trace element deficiency of this nature. The present studies do, however, confirm the earlier finding that both iron and copper are necessary to stimulate hemoglobin formation in the anemic condition produced.

SUMMARY

A copper deficiency produced in young growing pigs has been described. In addition to a dietary anemia, an unusual leg condition was encountered. There was lack of rigidity in the leg joints. The hocks became excessively flexed and forced the animal to assume a sitting position. The forelegs showed various types and degrees of crookedness. In the extreme state the use of the forelegs was also lost and the animal, although not paralyzed, remained in a prone position. Copper was shown to be therapeutic and in some cases brought about a complete reversal of symptoms.

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[6] Personal communication from Dr. M. O. Schultze.

PLATE 1

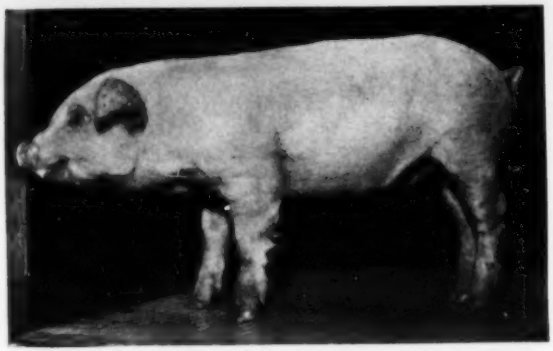
EXPLANATION OF FIGURES

2 Positive control. Fed 2 mg of copper per day.

3 Copper-deficient animal. Shows the drawing under of the rear and crookedness of the forelegs.

4 Copper-deficient animal, showing swelling in the region of the hocks and a turning of the rear leg.

5 Copper-deficient animal, showing the extreme weakness of the carpal joints in the foreleg.



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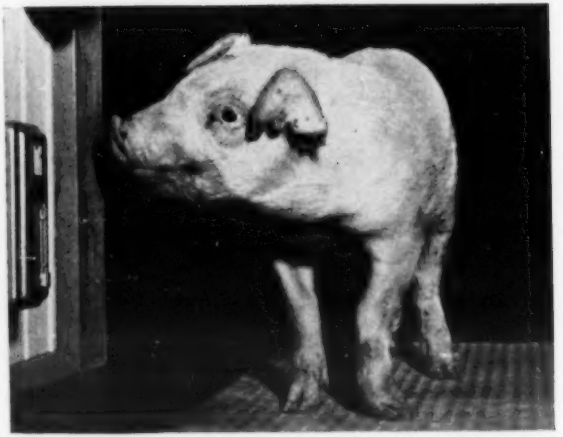

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THE DIGESTION AND ABSORPTION OF P^{32} LABELED CASEIN BY THE YOUNG CALF [1]

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THREE FIGURES

(Received for publication October 27, 1950)

Much reliable information can be found on the utilization of casein by the laboratory rat. On the other hand, very little data are available on the utilization of this important protein by the young calf. With the recent improvement in purified liquid diets (Wiese et al., '47) and the increasing availability of radioactive isotopes, there existed an opportunity to obtain some desired information as to the extent and mechanism of utilization of casein by the calf. The radioactive isotopes of nitrogen have half-lives of such short duration that they are of little value as tracers in studies of this sort. Casein, however, is a phosphoprotein and can be labeled with P^{32}, a radioactive isotope of phosphorus having a convenient half-life of 14 days (Kleiber et al., '48). Using casein labeled with P^{32}, research was begun to determine: (1) the rate of digestion of casein as measured by the appearance of the isotope in the blood stream; (2) the fate of the phosphorus present in the casein molecule during the process of digestion; (3) the distribution of isotopic phosphorus in the tissues of the calf's body; (4) the extent of digestion of the casein; and (5) the metabolic fecal protein excretion of the

[1] This work was supported in part by a grant from the Atomic Energy Commission.

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young calf under conditions of milk feeding. A preliminary report of this work has been presented (Lofgreen, '50). The present paper presents more detailed results on the first three objectives. Data on the latter two will be presented at a later date.

EXPERIMENTAL

Casein labeled with P^{32} was prepared by injecting lactating cows with from 40 to 100 millicuries of P^{32} as sodium phosphate in an isotonic saline solution. Injections were usually divided into 4 or 5 equal portions and made at 12-hour intervals. Milk was collected beginning after the second or third injection and continuing until such time as a sufficient quantity was obtained to yield the desired amount of casein. One part of skim milk was diluted with 4 parts of water and 0.1 part of 6% acetic acid was added with stirring. The precipitated casein was washed successively with water, alcohol and ether and allowed to dry. The casein prepared in this manner was incorporated into a purified diet similar to that described by Wiese et al. ('47).

Four calves between 9 and 10 weeks of age were given a single feeding of the diet made with the labeled casein. By means of venous catheterization similar to the method described by Ralston et al. ('49), blood samples were taken at frequent intervals following feeding. At 24 hours after feeding two of the calves were sacrificed and samples were taken for tissue distribution studies. For comparative purposes two similar calves were fed inorganic phosphate solution containing P^{32} by adding it to their milk for a single feeding. Blood samples were taken and the calves sacrificed 24 hours later to determine tissue distribution. Two more calves were injected every 20 minutes over a period of 24 hours with graded doses of inorganic phosphate solution containing P^{32} in an effort to duplicate as closely as possible the blood levels of P^{32} obtained by feeding labeled casein. Injections were made into one jugular vein and blood samples withdrawn from the other to avoid contamination. Blood and

tissue samples were taken as in the case of the other calves. A summary of data concerning the animals used and their treatment is presented in table 1.

All samples were dried, mixed with magnesium nitrate and ashed at 500°C. The ash was taken up in dilute HCl, keeping the volume small in order to maintain radioactivity at as high a level as possible. Radioactivity was determined by use of a Geiger counter and total phosphorus by the method of Fiske and Subbarow ('25). Because of the wide differences in dosages and body weights of the calves, the absolute blood and tissue levels were not directly comparable. Radioactive

TABLE 1

Description and treatment of experimental animals

CALF NO.	BREED [1]	AGE	WEIGHT	P^{32} ADMINISTERED	
				AMOUNT	METHOD
		days	*kg*	*μc*	
3	J	71	42.2	293	Fed in labeled casein
4	J	56	48.1	2760	Fed as inorganic salt
5	H	63	57.2	26.5	Fed in labeled casein
6	H	67	78.5	260	Injected as inorganic salt
7	H	68	68.5	72	Fed in labeled casein
8	G	63	54.5	60	Fed in labeled casein
9	H	67	73.5	1000	Fed as inorganic salt
10	G	67	45.4	1000	Injected as inorganic salt

[1] Guernsey, Holstein and Jersey are abbreviated as G, H and J, respectively.

phosphorus content is therefore expressed herein as corrected specific activity, which is equal to the microcuries (μc) of P^{32} per gram of phosphorus divided by the dose (in μc) per 100 gm body weight.

RESULTS AND DISCUSSION

The blood levels of radioactive phosphorus for 24 hours following feeding are presented in figures 1 and 2. Kleiber et al. ('50a, b) have shown that there is a continuous removal of labeled phosphate from the blood stream of cows. The rate of removal is dependent upon the level in the blood.

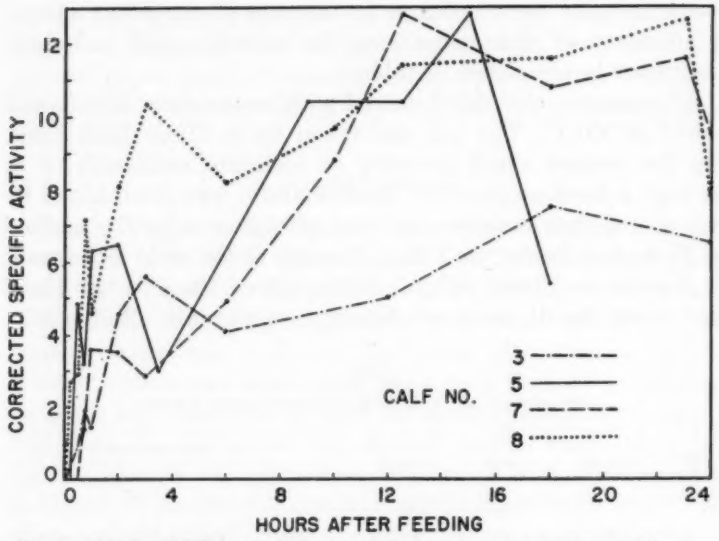

Fig. 1 Levels of P^{32} in the blood stream of calves fed labeled casein.

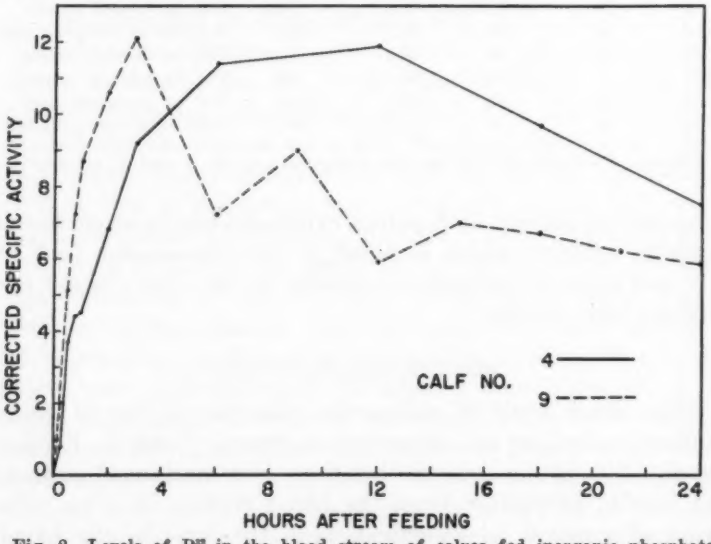

Fig. 2 Levels of P^{32} in the blood stream of calves fed inorganic phosphate in milk.

Changes which occur in the corrected specific activity in the blood of the calves fed P^{32} either in the form of casein or as inorganic phosphate would therefore reflect changes in absorption rate. For example, when the specific activity of the blood remains constant or increases despite this withdrawal, one must conclude that absorption is taking place at correspondingly different rates.

There is some variation in the curves representing the absorption of P^{32} from the labeled casein. There are, however, certain similarities. All 4 curves show an initial rise to a peak in from one to three hours, a fall and a more gradual rise to a second peak at from 12.5 to 23 hours. In two of the calves measurable blood levels of P^{32} were reached as early as 15 minutes after feeding. Animal phosphatases will not attack the phosphorus-amino acid linkage to any great extent in the intact casein molecule (Kay, '34). It appears, therefore, that within 15 minutes the proteinases and peptidases of the gastric, pancreatic and intestinal secretions may hydrolyze the casein molecule to the point where either the phosphate-containing substances can be absorbed or the phosphatases can attack the phosphate-amino acid linkage, releasing inorganic phosphate which is then absorbed. Even though the digestion process is initiated rapidly it continues for a long period, as is shown by the time at which the maximum levels are reached. This is in contrast to fat absorption by the calf, which reaches a peak during the first 6 hours and decreases to its initial level by the 12th hour (Barker and Jacobson, '49).

The blood levels of P^{32} presented in figure 2, obtained from the calves fed inorganic phosphate in their milk, are markedly different from those of the animals fed casein. This is as would be expected, since no digestion is necessary before the phosphate can be absorbed. It might be suspected from a comparison of the curves in figures 1 and 2 that the rather rapid rise to the first peak in the curves of the calves fed casein may have been due to the absorption of inorganic phosphate which had not been washed from the labeled casein

as it was prepared. In order to investigate this possibility the true protein was determined in a sample of casein by the method of Barstein (Wiegner, '26). The original casein contained 0.346 μc of P^{32} per gram, while the true protein prepared from 1 gm of casein contained 0.343 μc, showing that practically all the activity had been retained in the true protein fraction. It therefore seems reasonable to conclude that there was little contamination of the casein by inorganic phosphate and that the absorption curves in figure 1 represent the absorption of phosphorus which had been organically bound in the labeled casein.

During the process of digestion of the casein molecule there is undoubtedly some liberation of the phosphorus-containing compounds, of which phosphoserine may be the predominating one (Kay, '34). Damodaran and Ramachandran ('40) have presented evidence that an enzyme-resistant phosphopeptone containing glutamic acid, isoleucine and serine can be isolated from casein by subjecting it to the action of pepsin and trypsin. The possibility exists that some of these compounds may be absorbed unchanged into the blood stream. If significant amounts of phosphoserine or phosphopeptone are absorbed with the phosphorus remaining organically bound, it seems logical to expect that the tissue distribution of P^{32} in calves fed casein would be quite different from the distribution in those absorbing or having injected directly into the blood stream the phosphate in the inorganic form. A comparison of the tissue distribution of P^{32} between calves having similar blood levels over a 24-hour period but with the P^{32} coming in one case from injected P^{32} and in another case from digested casein should give some information concerning the absorption of the phosphorus in organic or inorganic form. Such a comparison is presented in figure 3. The corrected specific activities for the tissues of the calves fed either casein or inorganic phosphate are, with the exception of the organs of the gastrointestinal tract, consistently lower than those of the injected calves because of the incomplete absorption from the digestive tract. The specific activity values have been

corrected for the total dose per unit of body weight, while the dose actually reaching the tissues of the calves fed casein or inorganic phosphate would be somewhat less than the total

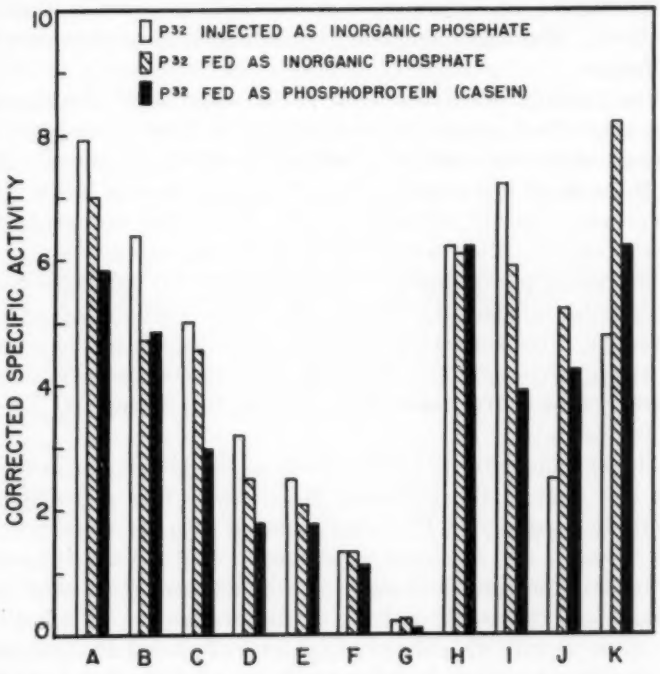

Fig. 3 Distribution of P^{32} in representative tissues of calves fed either labeled casein or labeled inorganic phosphate or injected with labeled inorganic phosphate. A, liver; B, kidney; C, heart; D, lung; E, testes; F, skeletal muscle; G, brain; H, rumen; I, omasum; J, abomasum; K, jejunum.

consumed. If it were possible to correct for the incomplete absorption the levels would be more nearly equal.

The distribution pattern, excluding the tissues of the gastrointestinal tract, is very similar among the three methods of administration. Since it would be logical to expect a different pattern of distribution of phosphorus organically

bound to an amino acid and inorganic phosphate not so bound, the results of this study give no evidence that phosphoserine or phosphopeptone are absorbed as such. The liver showed the highest rate of phosphate exchange in all except one calf, in which the exchange rate of the kidney equaled that of the liver. The brain consistently showed a very slow rate of exchange.

The pattern of distribution in the organs of the gastrointestinal tract seems to be different in that it appears to depend upon the method of administration. It is quite difficult to wash the organs free of contamination when they have been directly in contact with either the inorganic P^{32} or the casein. This would occur with the calves fed casein or inorganic phosphate and could account for the differences in distribution pattern among the three methods of administration. The measurement of the distribution in the organs of the gastrointestinal tract of the injected calves should therefore be more valid than that in the calves fed P^{32} in either form.

The specific activity of the tissue of the abomasum is about one-half that in the jejunum. Since there was a practically continuous supply of P^{32} being injected into the blood stream for 24 hours, and since one must assume that the specific activity before injection was zero, it is probable that the tissues possessing a high specific activity at the termination of injections are those tissues which have a high rate of phosphate exchange. At lengthening periods after cessation of P^{32} administration this assumption may not be true, since the organs with the more rapid exchange rate would lose their P^{32} content more rapidly than those exhibiting a slower rate. Because the calves were sacrificed just at the termination of the injections, it seems justifiable to consider that the tissues having a high specific activity also possess a high rate of phosphate exchange. It appears, therefore, that the abomasum exhibits a much slower rate of phosphate exchange than does the jejunum. There is considerable evidence that rapid phosphorylation and dephosphorylation mechanisms are important

in the absorption of certain nutrients (Somogyi, '42; Deuel, '43; Evans, '44). It is possible that the more rapid rate of phosphate exchange in the small intestine may be due in part to the importance of this organ in absorption processes. In order to determine if the same relationship between the distribution in the tissues of the stomach and the small intestine 24 hours after injection would hold true in other animals, comparisons were made from data available at this institution. A summary of such data is presented in table 2.

The specific activity of the tissue of the small intestine in all cases is almost twice as high as that in the abomasum.

TABLE 2

Specific activity of the tissues of the small intestine and true stomach of different animals sacrificed 24 hours after injection of P^{32}

ANIMAL	μC P^{32} PER GRAM OF P		
	SMALL INTESTINE	TRUE STOMACH	RATIO
Rabbit	260	167	1.6
Hog	295	171	1.7
Sheep	76	47	1.6
Calf	33	18	1.8
Cow	20	10	2.0

In table 3 are presented the ratios of the specific activities of the various organs of the digestive tract to those of the abomasum of the two injected calves, 6 and 10.

In both calves all organs had a higher specific activity, and thus a higher rate of exchange, than the abomasum, with the omasum, rumen and jejunum ranking highest. The rapid rate of exchange in these organs may be due to either phosphorylation mechanisms involved in the absorption of nutrients, phosphorylation processes concerned in the metabolism of the tissue itself, or merely the transport of phosphate from the blood to the gastrointestinal contents. As was previously pointed out, phosphorylation mechanisms may be important in absorption. On the basis of the oxygen consumption of intestinal tissue in comparison to that of the stomach, one

might expect this organ to exhibit a rapid rate of phosphate exchange. Carlyle ('48) reported that the intestine of sheep consistently exhibits a higher Q_{O_2} than the stomach. Dickens and Weil-Malherbe ('41) state that the mucous membrane of the jejunum is among the most actively respiring tissues of the body. The possibility exists that the rapid exchange rates of the omasum and rumen may be partially explained on these bases. Barcroft et al. ('44) have shown the rumen to be important in the absorption of organic acids. Wildt (1874) noted that phosphorus secretion exceeded absorption in the rumen and abomasum, while absorption exceeded secretion in the

TABLE 3

Ratio of the specific activity of the tissues of the gastrointestinal tract to that of the abomasum of calves injected with P^{32}

ORGAN	CALF	
	6	10
Rumen	2.9	1.9
Omasum	2.9	2.8
Jejunum	2.0	1.8
Cecum	1.8	1.2
Colon	1.7	1.6
Rectum	1.5	1.5
Abomasum	1.0	1.0

omasum and small intestine of sheep. It appears doubtful that the transport of phosphate from the blood through the rumen wall to the contents could account for the high activity of the rumen tissue, especially when one considers that the abomasum exhibits a slow rate of exchange and that phosphate is also transferred from blood to contents in this organ. This factor certainly cannot account for the high specific activity of the omasum of the injected calves, since absorption exceeds secretion in this part of the tract. Further work must be done to determine the significance of the phosphate exchange rates with respect to the role of these organs in the absorption process.

SUMMARY

Studies are reported on the rate of absorption of P^{32} by calves fed labeled casein, and comparisons are made with similar calves fed or injected with the isotope in inorganic form. Tissue distribution of the P^{32} was determined 24 hours after feeding.

The results show that the digestion of casein is initiated rather rapidly and is prolonged over the 24-hour period. When labeled inorganic phosphate is added to the milk, absorption takes place in a much different pattern.

Tissue distribution of the isotope indicated that it is probable that the calf absorbs inorganic phosphate even when it is fed in the organic form as phosphoprotein. Liver and kidney consistently showed rapid phosphate exchange rates, while brain tissue rates were notably slow. The omasum, rumen and jejunum appeared to have high phosphate exchange rates in comparison to other sections of the gastrointestinal tract.

ACKNOWLEDGMENTS

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THIAMINE DEFICIENCY IN THE LAMB

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THREE FIGURES

(Received for publication November 6, 1950)

The synthesis of some members of the vitamin B complex by the ruminant was demonstrated by McElroy and Goss ('39). Development of a "synthetic" milk diet for calves by Wiese, Johnson, Mitchell and Nevens ('47) facilitated the study of individual vitamin deficiencies in the young ruminant, and the results of several such investigations have been reported. Garrigus and Johnson ('49) have succeeded in raising newborn lambs on this diet, and Luecke and co-workers ('50) have employed a similar "synthetic" milk diet in producing a riboflavin deficiency in this species. Johnson et al. ('48a) demonstrated the essentiality of thiamine for the dairy calf. In this paper the effect of deleting thiamine from the diet of the lamb is reported.

EXPERIMENTAL

Six crossbred lambs one to three days old were selected for the study. Prior to being placed on experiment they had all received colostrum. The animals were housed in wire cages with raised floors to permit urine collection. They were fed a "synthetic" milk diet by bottle, and the ad libitum food consumption was recorded.

The diet employed was of the composition shown in table 1. This diet was made up to contain 13% solids, as described in previous reports from this laboratory. The water-soluble vitamins were taken up in a 30% ethanol solution and were

added to the milk at feeding time. In addition, each lamb received 10,000 I. U. of vitamin A and 2,000 I. U. of vitamin D at the start of the experiment. Sulfathalidine was added to the milk at a rate of 3 gm per liter.

Thiamine was deleted from the diet of 4 of the lambs; the remaining two were retained as positive controls. Weekly

TABLE 1

Composition of synthetic diet

CONSTITUENTS	AMOUNT
Casein, "vitamin-free" (%, dry basis)	30
Lard (%, dry basis)	30
Mineral mixture[1] (% dry basis)	6
Cerelose (%, dry basis)	34
Water-soluble vitamins (mg/1 of milk):	
Thiamine hydrochloride	0.65
Riboflavin	1.30
Calcium pantothenate	1.95
Nicotinic acid	2.60
Pyridoxine	0.65
Inositol	26.0
Choline	260.0
Folic acid	0.26
Biotin	0.02
Fat-soluble vitamins (quantity/1 of milk):	
Vitamin A	2,000 I.U.
Vitamin D	200 I.U.
α-Tocopherol	1.0 mg
2-Methyl-1, 4-naphthoquinone	0.26 mg

[1] For composition of mineral mixture, see Johnson, James and Krider ('48b).

urine collections were made for thiamine and pyruvic acid determinations.

RESULTS AND DISCUSSION

The lambs receiving thiamine in their diet grew at rates of 0.35 lb. and 0.43 lb. per day over experimental periods of 6 and 5 weeks, respectively. At the conclusion of the test their appearance was considered normal.

The development of the symptoms of thiamine inadequacy in the remaining 4 lambs followed a uniform and characteris-

tic pattern marked by anorexia and growth stoppage after two to three weeks, followed in the 4th to 5th week by convulsive seizures, including opisthotonos. The growth curves for the two positive controls (1 and 2) and 4 deficient animals (3, 4, 5 and 6) are shown in figure 1.

Lamb 3 gained 0.38 lb. daily during the first two weeks of the experiment, and consumed an average of 1,160 ml of milk. In the third week the appetite declined, and no gain in weight was obtained. By the start of the 4th week food consumption had declined to approximately 200 ml daily, and the lamb showed signs of emaciation, listlessness and dehydration. On the 25th day there developed a convulsion of the type considered typical of thiamine deficiency in other species. These tetanic spasms were at first intermittent, and consisted primarily of spasmodic retractions of the head which caused the animal to fall back into a sitting position. As the seizures increased in severity, the lamb toppled over from the sitting posture and entered into prolonged periods of convulsions, chiefly characterized by retraction of the head and thrashing of the legs.

After the onset of severe convulsions this lamb was given 10 mg of thiamine hydrochloride intramuscularly. Ninety minutes later the spasms disappeared, and within a few hours the appetite was greatly improved. During the succeeding 10 days thiamine was included in the diet and growth was resumed at an average rate of 0.77 lb. per day.

A similar sequence of symptoms occurred in the case of lamb 5, which developed convulsions on the 27th day of being fed the thiamine-deficient diet (see fig. 2). A heart rate of 50 to 60 per minute was recorded during the quiescent periods between seizures, indicating that, like the rat but unlike the human, the lamb undergoes bradycardia in thiamine deficiency.

With the possibility in mind of temporarily relieving the convulsions by counteracting any possible effect due to low food intake, 20 ml of 5% glucose solution were administered intravenously. An immediate and marked response was ob-

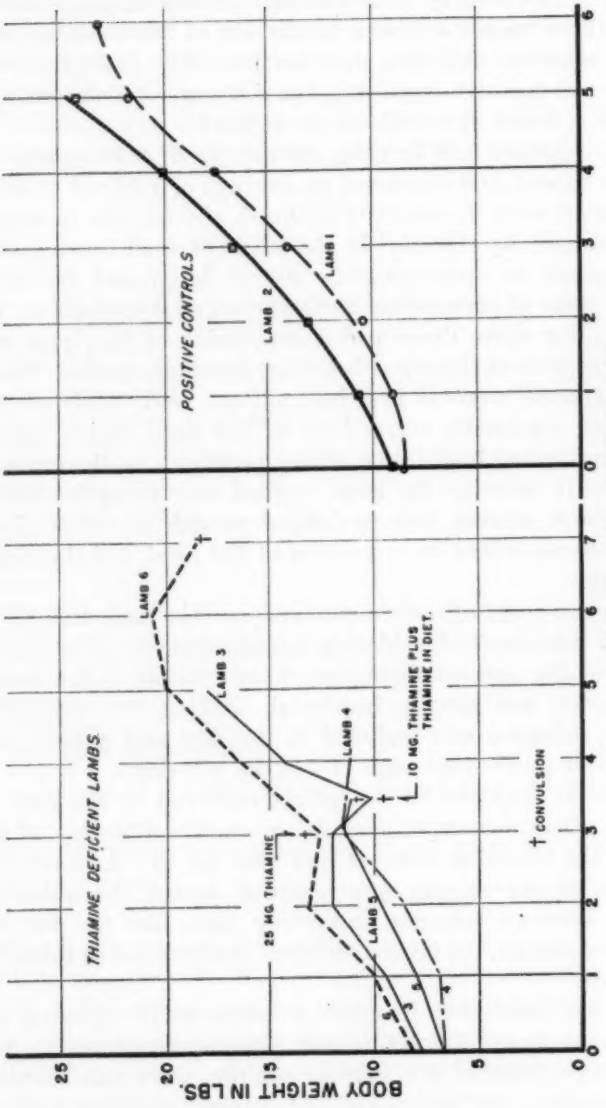

Fig. 1 Growth curves of thiamine-deficient lambs and positive controls.

tained. The lamb showed symptoms of intense muscular contraction throughout the body, including extension of the head and protrusion of the tongue, accompanied by evidence of severe pain. These symptoms culminated in death within two minutes after the injection of glucose.

Post mortem examination of lamb 5 did not reveal any evidence of heart enlargement or of significant lesions of the heart or sciatic nerve. Lack of neurological symptoms in the

Fig. 2 Lamb 5 in a state of acute thiamine deficiency.

presence of acute thiamine deficiency has been reported previously by Wintrobe et al. ('42) and by other workers.

Lamb 4 manifested the usual symptoms of deficiency, including convulsions on the 30th day of thiamine depletion. The marked opisthotonos exhibited by this animal is shown in figure 3. A heart rate of 65 to 70 per minute was recorded between spasms. Glucose was also administered to this lamb, but, following the experience with lamb 5, the quantity was reduced to 0.5 gm given in solution subcutaneously to reduce

the rate of uptake. After 20 minutes the same picture of muscular contraction, straining and acute pain as was observed in lamb 5 developed, and the animal subsequently died. Examination of lamb 4 revealed no significant histopathological abnormality.

Lamb 6 exhibited a typical convulsion after 23 days on the thiamine-deficient diet. Following the administration of 25 mg of thiamine intramuscularly this lamb underwent complete recovery from the seizures within half an hour. Subsequently the animal was retained on the deficient diet and gained 0.53 lb. daily over the succeeding two-week period.

Fig. 3 Lamb 4 exhibiting opisthotonos as a result of thiamine deficiency.

Reports in the literature on the toxicity of high levels of pyruvic acid administered to mice and rats describe symptoms strikingly similar to those exhibited by these lambs in a state of acute thiamine deficiency. Dobrovolskaïa-Zavadskaïa ('46) has described a condition produced in normal mice by administering massive doses of sodium pyruvate which was marked by opisthotonos, forced respiration, sharp pain and

general convulsion. Butturini ('40) found that the LD 50 of sodium pyruvate for mice and rats was between 4 and 5 gm per kilogram of body weight. These observations suggest that the acute muscular symptoms obtained in thiamine deficiency may be more accurately attributed to the toxic accumulation of pyruvic acid, in the decarboxylation of which thiamine is involved, than to the depletion of thiamine per se.

The knowledge that the aforementioned reactions can be obtained in the animal in a normal state of thiamine nutrition by the use of large amounts of pyruvate (for example, 0.1 gm of sodum pyruvate for a 12-gm mouse in the first report cited) suggested that relatively smaller doses might be effective in animals whose tissues already contained subnormal concentrations of thiamine and whose blood contained supernormal amounts of pyruvic acid. Consequently, lamb 6, after having been maintained on the thiamine-deficient diet for 15 days following the administration of 25 mg of the vitamin, was slowly given 1 gm of sodium pyruvate intravenously. An immediate, but transitory, effect was obtained which resembled the onset of a convulsive seizure as described earlier. The animal displayed spasmodic head retractions, assumed a sitting posture and showed evidence of incoordination. These symptoms passed within one minute. Similar responses were obtained on the 17th and 22nd days of thiamine deprivation. On the 30th day the lamb developed convulsions for the second time without the administration of pyruvate. After having failed to respond to 1 gm of sodium fumarate given subcutaneously and orally, the animal recovered after 7 hours in nearly continuous seizures when treated with 50 mg of thiamine intramuscularly.

Twenty-four-hour excretions of thiamine and pyruvic acid were determined weekly. The thiamine assay procedure employed was that of Sarett and Cheldelin ('44) using *L. fermenti 36*. Pyruvic acid was determined by the method of Friedemann and Haugen ('43). The results of the thiamine assays are shown in table 2.

The wide differences in the urinary excretion of thiamine between the two groups of lambs are evident from table 2. In the deficient animals the excretion of the vitamin declined to only trace amounts.

The data for pyruvic acid excretion indicated that a sharp increase in urinary concentration occurred immediately prior to the development of convulsions. Thus, the urine of lamb 6, on the day before the occurrence of seizures, contained 3.8 mg % of pyruvic acid. By contrast, that of both positive controls contained 1.1 mg % after a similar period of feeding. The urine of the three remaining deficient lambs contained 1.3, 1.0 and 1.8 mg % pyruvate, two, 6 and three days before the onset of convulsions, respectively. These data also comprise supporting evidence of a close relationship between the occurrence of spasms and pyruvic acid accumulation.

TABLE 2

Twenty-four-hour excretion of thiamine by deficient and control lambs (μg)

LAMB NO.	WEEKS ON EXPERIMENT					
	0	1	2	3	4	5
Positive controls						
1	23	34	25	21	43	38
2	25	15	21	25	34	..
Deficient lambs						
3	33	5	8	7	..	..
4	24	3	3	1	2	..
5	18	6	10	1	..	..
6	41	14	6	3	..	..

SUMMARY AND CONCLUSIONS

The performance of a group of 4 lambs, one to three days old, fed a "synthetic" milk diet deficient in thiamine, was compared to that of two positive controls. Those receiving the deficient diet developed anorexia, emaciation and somnolescence after about three weeks, followed by tetanic con-

vulsions. These symptoms were rapidly abolished by the administration of thiamine. Transitory convulsive spasms were also obtained by injecting sodium pyruvate. The lambs receiving the same diet with thiamine added grew satisfactorily and appeared normal.

The results obtained in this experiment support the conclusion that thiamine is an essential nutrient for the lamb.

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SUPPLEMENTAL VALUE OF CERTAIN AMINO ACIDS FOR LAMB PROTEIN AND NUTRITIVE VALUE OF PROTEIN IN DIFFERENT CUTS OF LAMB

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The annual per capita consumption of lamb and mutton in the United States in 1949 was 4.1 pounds as compared with 143.9 pounds of all kinds of meat, or 2.85% of the total (U. S. Department of Agriculture, '50). As regards the nutritive value of lamb protein, available information indicates that the protein in lamb is similar in amino acid content and in biological value to the protein in other kinds of muscle meat. Schweigert et al. ('49) report the determination of 10 essential amino acids in pork and lamb cuts by microbiological methods. With the exception of histidine, the values for lamb are similar to those for pork. Numerous references to previous investigations concerning the amino acid content of lamb and other meats are cited.

The biological or growth-promoting value of lamb protein has not been studied extensively. Hoagland and Snider ('26, '46), found the protein in lamb to be similar in nutritive value to that in beef and pork.

The purposes of the experiments herein reported were to determine: (1) whether lamb protein is deficient in cystine, methionine, leucine, isoleucine, phenylalanine, tryptophan, or valine and; (2) the biological value of the protein in different cuts of lamb.

EXPERIMENTAL

Products tested

Two lots of lambs were used in the experiments. The lambs were raised at the Agricultural Research Center, Beltsville, Maryland. The first lot was slaughtered in October, 1948, and the second lot in January, 1950. The dehydrated lean meat from lot 1 was used in experiments 1 and 2 which are reported in tables 1 and 2; and the lean meat from lot 2 was used in experiment 3, which is reported in table 2.

The lean meat from regular cut legs, square cut shoulders, and entire carcasses was trimmed as free from fat as practicable, cut into small pieces, frozen at 0°F. and ground. The meat was then dehydrated in a current of air at a temperature of 120°F. to a moisture content of less than 10%. The dehydrated meat was thoroughly extracted with ethyl ether at room temperature, ground fine and stored in tightly covered tin cans at approximately 20°F.

Diets fed

In experiment 1 (table 1) the diets were prepared as follows: Dehydrated lamb from the entire carcass was incorporated in each diet in such quantity as to supply 1.2% nitrogen, or 7.5% protein. The following amino acids were added in the quantities indicated: L-cystine, and the DL-forms of methionine, leucine, isoleucine, phenylalanine, tryptophan, and valine. When the diets contained added leucine, isoleucine, or valine, only one-half of the nitrogen content of each was charged against the diet, since only the natural isomers of these amino acids are utilized by the rat.

The B vitamins were mixed with dextrin in such quantities that 2.5 gm of the mixture supplied the following quantities in milligrams per 100 gm of diet: thiamine hydrochloride, 0.3; riboflavin, 0.3; pyridoxine hydrochloride, 0.6; calcium pantothenate, 1.5; and choline chloride, 40.

The fat-soluble vitamins were mixed with lard in such quantities that 1 gm of the mixture contributed the following quantities to 100 gm of diet: 5 I. U. of vitamin A, 1 unit of

TABLE 1

Supplemental value of certain amino acids for lamb protein, using meat from the entire carcass, at the 7.5% level in 30-day tests with groups of 8 male albino rats

Diet no.	DESCRIPTION OF DIETS: Amino acids added to diets		Nitrogen in diets	GAIN IN WEIGHT	FEED CONSUMED	FEED CONSUMED PER GRAM GAIN	GAIN PER GRAM OF NITROGEN
		%	%	gm	gm	gm	gm
			Experiment 1				
1			1.200	83	300	3.61	23.1
2[1]	L-cystine	0.150	1.217	115	342	2.97	27.4
3	L-cystine	0.225	1.226	111	330	2.97	27.3
4	DL-methionine	0.150	1.214	113	331	2.93	28.1
5	DL-methionine	0.225	1.221	112	338	3.11	27.2
6	DL-methionine DL-phenylalanine	0.150 0.100	1.222	109	337	3.09	26.5
7	DL-methionine DL-leucine	0.150 0.220	1.226	105	324	3.09	26.3
8[1]	DL-methionine DL-isoleucine	0.150 0.260	1.228	109	337	3.09	26.2
9	DL-methionine DL-valine	0.150 0.240	1.229	111	323	2.91	27.2
10	DL-methionine DL-tryptophan	0.150 0.014	1.216	112	333	2.91	27.6
11	DL-methionine DL-phenylalanine DL-leucine DL-isoleucine DL-valine DL-tryptophan	0.150 0.100 0.220 0.260 0.240 0.014	1.264	79	274	3.47	22.6

[1] Seven rats.

TABLE 2

Nutritive value of protein in different cuts of lamb in 30-day tests with groups of 8 male albino rats

DESCRIPTION OF DIETS					GAIN IN WEIGHT	FEED CONSUMED	FEED CONSUMED PER GRAM GAIN IN WEIGHT	GAIN IN WEIGHT PER GRAM OF NITROGEN
Diet no.	Lamb cut	Laboratory no.	Protein content	Nitrogen content				
			%	%	*gm*	*gm*	*gm*	*gm*
				Experiment 2				
1A	Leg	4538	7.5	1.2	76	290	3.82	21.9
2A	Shoulder	4539	7.5	1.2	88	310	3.52	23.5
3A	Entire carcass	4540	7.5	1.2	83	300	3.61	23.1
	Average				82	300	3.65	22.8
4A	Leg	4538	10.0	1.6	111	329	2.96	21.1
5A	Shoulder	4539	10.0	1.6	128	363	2.84	22.1
6A	Entire carcass	4540	10.0	1.6	131	362	2.77	22.6
	Average				123	351	2.86	21.9
7A	Leg	4538	12.5	2.0	144	362	2.51	20.0
8A	Shoulder	4539	12.5	2.0	142	357	2.51	19.9
9A	Entire carcass	4540	12.5	2.0	159	379	2.38	20.9
	Average				148	366	2.47	20.3
				Experiment 3				
10A	Leg	4598	12.5	2.0	98	281	2.87	17.4
11A	Shoulder	4597	12.5	2.0	98	288	2.94	17.4
12A	Entire carcass	4596	12.5	2.0	105	296	2.82	17.7
	Average				100	288	2.88	17.5
13A	Leg	4598	15.0	2.4	122	298	2.44	17.0
14A	Shoulder	4597	15.0	2.4	127	313	2.48	16.8
15A	Entire carcass	4596	15.0	2.4	126	311	2.48	16.9
	Average				125	307	2.47	16.9

vitamin D, and 20 μg of alpha-tocopherol. Sufficient lard was added to make 10% fat in the diet. Salt mixture amounting to 4% and dextrin to make 100% completed the diet.

In experiment 2, the diets were prepared in the same manner except that the different lamb cuts were added in such proportions as to supply 7.5, 10.0, and 12.5% of protein, and no amino acids were added.

In experiment 3, the lamb cuts were added to the diets in such quantities as to supply 12.5 and 15% of protein, and the following additional vitamins were added in milligrams per 100 gm of diet: niacin, 5; inositol, 20; para-aminobenzoic acid, 7.5; biotin, 0.02; folic acid, 0.04; liver extract, 375; and 2-methyl naphthoquinone, 0.2.

Each diet was made up in the quantity of 1 kg at one time and was stored in a refrigerator at 40°F. Each diet was fed to 8 male rats weighing approximately 40 gm each and not exceeding 24 days of age. The rats were kept in individual cages with raised screen bottoms, self-feeders, and drinking bottles. The bottom of each cage was covered with a sheet of blotting paper. The rats were weighed twice weekly and the feeders were refilled about every third or 4th day. The temperature of the rat laboratory was maintained at approximately 75°F.

RESULTS

Experiment 1

The results of the feeding experiment with young male rats to determine the effects of the addition of 6 essential amino acids and of cystine to diets containing 7.5% protein from the entire carcass of a lamb are summarized in table 1.

A statistical analysis of the experimental data was made by means of Fisher's "t" values for the significance of differences between the means of the gain in weight per gram of nitrogen consumed. A comparison of the biological or growth-promoting value of the protein in diet 1, which contained no added amino acid, with the values for diets 2 to 10, inclusive, to which various amino acids had been added, shows

that all of these differences are highly significant (< 0.001). The difference between the values for diets 1 and 11 is not significant.

A further examination of the data in table 1 shows that the addition of 0.15% of either cystine or methionine to the basal diet induced a marked increase in the rate of growth of the rats and in the efficiency with which the protein was utilized. The addition of a larger quantity of either amino acid was without further effect. When 0.15% of methionine was fed in combination with either phenylalanine, leucine, isoleucine, valine or tryptophan, in no case was the gain in weight of the rats, or the biological value of the protein, greater than the values for the diet to which methionine alone had been added.

The results obtained with diet 11, to which 6 essential amino acids had been added, are peculiar in that the rate of growth of the rats and the biological value of the protein were practically the same as for the basal diet alone. The reason for this result is not apparent.

The results of experiment 1 indicate very clearly that the protein in the dehydrated lean meat from the entire carcass of a lamb, when fed at the 7.5% level for 30 days to young male albino rats, was deficient only in either cystine or methionine of the amino acids tested. When either of these amino acids was added to the diet as the sole nitrogen supplement, the nutritive value of the lamb protein was greatly increased, and further additions of combinations of amino acids did not enhance the value.

These results are very similar to those previously reported by Hoagland et al. ('48) in a similar study of the supplemental value of the same group of amino acids for beef protein.

Experiment 2

In table 2 are summarized the results of experiment 2 to determine the relative nutritive values of the protein in the lean meat from the leg and shoulder cuts and from the entire

carcass of lambs when protein was fed at the 7.5, 10.0, and 12.5% protein levels.

A statistical analysis was made for the significance of the differences between the means of the gain in weight per gram of nitrogen consumed, with the following results: diet 1A with diet 2A, highly significant ($\angle 0.01$); diet 1A with diet 3A, significant ($\angle 0.02$); diet 4A with diet 5A, not signficant; diet 4A with diet 6A, highly significant ($\angle 0.001$); diet 7A with diet 8A, not significant; diet 7A with diet 9A, highly significant ($\angle 0.01$); and diet 8A with diet 9A, significant ($\angle 0.05$).

The results of experiment 2 indicate that the growth-promoting value of the protein in the leg of lamb was significantly lower at each level of protein intake than the protein in the entire carcass. The protein in the leg was significantly lower in value than that in the shoulder only at the 7.5% protein level. When the diets contained 12.5% protein, the protein in the shoulder was of slightly lower growth-promoting value ($\angle 0.05$) than that in the entire carcass. In general, the results of experiment 2 indicate a superiority in the nutritive value of the protein in the entire carcass of lamb as compared with the protein in the leg.

Experiment 3

Experiment 3 was conducted about a year later than experiment 2 with a different lot of lamb carcasses. The results of this experiment also are presented in table 2. The dehydrated lean meat from the leg, shoulder and entire carcass of lambs was fed at the 12.5 and 15% levels of protein intake. The data for this experiment show practically no differences between the growth-promoting values for the protein in the three cuts of lamb at either level of protein intake. These results are somewhat different from those in experiment 2, when the protein in the entire carcass was found to be superior in growth-promoting properties at the 7.5, 10.0, and 12.5% protein levels.

SUMMARY

Experiments were conducted with young male albino rats to determine: (1) whether lamb protein is deficient in cystine, methionine, leucine, isoleucine, phenylalanine, tryptophan or valine at the 7.5% protein level; and (2) the biological value of the protein in different cuts of lamb at 4 levels of intake.

The results of these experiments indicate that the protein from the entire carcass of lamb is deficient only in cystine or methionine. The addition of the same quantity of either amino acid to the diet was equally effective in promoting much more rapid growth and better utilization of the lamb protein.

When the protein in the leg, shoulder, and entire carcass of lamb was fed at the 7.5, 10.0, 12.5, and 15.0% levels of intake, it was found in one experiment, at the first three protein levels, that the protein in the entire carcass was superior in biological value to that in the leg. In a second experiment with another lot of lambs, when protein constituted 12.5 and 15.0% of the diets, practically the same biological values were obtained for the three cuts of lamb at each protein level.

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THE EFFECT OF HEAT TREATMENT ON THE NUTRITIONAL VALUE OF SOME VEGETABLE OILS

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INTRODUCTION

Oil-seed crops are becoming increasingly important to Canadian economy. While the meal plays a significant role in livestock feeding, the oils pressed from seeds have been adapted to many uses. Of current concern is the manufacture of vegetable oils into edible products such as margarine and salad oils.

Because certain of the more highly unsaturated vegetable oils develop undesirable odors and flavors on exposure to heat or light, their use as edible products was hindered. In 1945 Privett and his co-workers showed thermal polymerization to be a simple and yet satisfactory method for rendering such vegetable oils less susceptible to this "flavor reversion." Polymerization has reportedly been used in the processing of herring oil for human consumption in the United States (Lassen et al., '49), and in Norway (Schwitzer, '48) to replace olive, cotton seed and groundnut oils with herring oil for canning, cooking and to some extent as a table and salad oil.

However, when linseed oil prepared by the method of Privett et al. ('45) was fed in 28-day tests to rats in this laboratory (Crampton and Millar, '46) a high incidence of death occurred.

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Because unheated linseed oil is known to be wholesome and nutritious (Molotkow, '32), it was suspected that heat polymerization had somehow deleteriously affected the nutritional value of that oil.

Reports in the literature of adverse affects on fats of severe heat are scanty. Roffo ('44) found that rats fed sunflower seed and olive oils heated to 350°C. for 30 minutes developed stomach tumors which became cancerous over a period of 12 to 30 months. Although no other published reports confirm these observations, Morris et al. ('43) noted growth failure and weight loss in rats fed diets containing 50% lard which had been heated at 300°C. for 120 minutes. Accompanying these effects on growth, Morris observed in some animals a paralysis similar to that produced in rats fed a vitamin E-deficient diet by Mackenzie et al. ('40); also some cirrhosis of the liver and chronic gastric ulcers. However, both these groups of workers used fat heated in air, where oxidation may have been an important factor, whereas Privett's polymerization is conducted in an atmosphere of inert gas.

In 1947, Harris fed rats fish oils heated at 280°C. under a vacuum of 10 mm of mercury for 8 to 12 hours and observed symptoms of retarded growth and general ill-health.

Subsequent to our original findings, a series of experiments was conducted, the most pertinent of which are reported herein, to investigate further the influence of heat on the nutritiousness of herring oil, linseed oil and other vegetable oils which are potentially important to the food industry.

EXPERIMENTAL PROCEDURE

The general plan of each of the tests in this study involved feeding to young growing rats basal diets in which one or the other of the several fats, subjected to heats of varying duration, was incorporated at levels of 10% or 20% by weight. Fats examined in the tests reported include linseed, soybean, rapeseed, corn, peanut, and herring oils.

The vegetable oils were heated in a salt bath at 275°C. for the desired time in all-glass apparatus. Carbon dioxide was blown through the oil continuously to exclude air and to remove volatile decomposition products thoroughly. The herring oil was a caustic soda-refined, bleached, deodorized product of a process reportedly used in Norway (Schwitzer, '48), involving heating while passing through steam at 280°C. under a vacuum of 10 mm of mercury for 10 hours.

The effect of the heat treatment on the nutritive value of the oils was measured by the growth rates of the rats, their feed consumption, and their general conditions of health and thrift as compared to those of animals fed identical basal diets but containing the unheated oil. Post mortem examination of the viscera was performed on all rats failing to survive the planned feeding period and on representatives of the groups at the close of the test.

Equal numbers of male and female white rats of approximately 28 days of age were allotted at random within sexes to the test groups, which ranged in size from 6 to 16 animals per lot. Thus each lot contained equal numbers of male and female animals. They were housed in individual wire-bottom cages, and were allowed feed and water ad libitum throughout each 28-day test period. Weekly weight gain and feed consumption for each rat were recorded.

Diets

The percentage compositions of the three basal diets used are shown in table 1.

It will be noted that diets I and III differed only in the level of fat, which varied by weight at the expense of the flour. Diet II was a modification of diet I through the addition of yeast in place of the thiamine, riboflavin and niacin. The level of protein in the basal diets was such that it supplied between 20% and 24% of the Calories.

In preparation for feeding, the oils were added to the thoroughly mixed dry ingredients and the whole baked at 375°F.

for approximately 20 minutes. The resulting biscuit was then granulated and air-dried for 24 hours, after which the supplements were added. Diets were refrigerator-stored until the beginning of the test period, during which time they were held at room temperature.

In addition to the diet, weekly doses of cod liver oil were administered orally to supply 175 I.U. of vitamin A and 35 I.U. of vitamin D.

TABLE 1

Percentage compostion of the basal diets

INGREDIENTS	I	II	III
White flour	47.0	44.0	57.0
Casein	11.5	11.5	11.5
Milk powder	19.0	19.0	19.0
Oil	20.0	20.0	10.0
Bone meal	2.0	2.0	2.0
Salt	0.5	0.5	0.5
Yeast [1]	..	3.0	..
Thiamine [1]	10 p.p.m.	..	10 p.p.m.
Niacin [1]	230 p.p.m.	..	230 p.p.m.
Riboflavin [1]	5 p.p.m.	..	5 p.p.m.
Protein in the diet (%)	20.4	20.4	24.3

[1] Added after baking.

RESULTS OF FEEDING TRIALS

Clinical observations

Symptoms of defective diet were most pronounced in those animals fed polymerized linseed oil, and the severity of the symptoms varied with the amount of oil incorporated in the diet. However, animals fed any of the polymerized oils, and whose weight gains were significantly lower than those of their controls, excreted dark, sticky feces. Lassen et al. ('49) noted abnormal feces from adult rats fed polymerized sardine oil as 5% of the diet. In our tests, in addition to the sticky fecal residue, animals fed polymerized oil as 20% of the diet showed oily, matted coats.

Growth and feed consumption

In examination of the results which follow it must be understood that the data presented were collected from a series of individual tests. Control groups of animals fed the appropriate unheated oils were carried in each experiment, and quantitative comparisons of the data shown in these tables are strictly valid only within tests.

TABLE 2

Summary of data showing the effects of heat treatment of oils as measured by daily gains of rats, feed intakes, and gains per 1,000 Calories eaten

TEST AND OIL USED	DIET NO.	OIL IN DIET	DURATION OF HEAT TREATMENT AT 275° C.	NO. OF ANIMALS	AVE. DAILY GAIN	AVE. DAILY FEED INTAKE	GAIN/ 1,000 CALORIES
		%	hrs.		gm	gm	gm
Linseed	III	10	nil	10	3.9	9.6	91
		10	2	10	3.8	9.6	91
		10	4	10	3.4 [1]	9.5	80
		10	8	10	2.5 [2]	8.1	69
Rapeseed	III	10	nil	10	3.4	10.0	76
		10	15	10	2.9	9.5	69
		10	30	10	2.3 [1]	7.8	64
Corn	III	10	nil	12	3.6	10.4	78
		10	15	12	2.8	8.7	71
		10	30	12	2.3 [1]	8.0	64
Peanut	III	10	nil	12	3.6	9.3	87
		10	15	12	3.6	10.0	80
		10	30	12	0.8 [1]	5.2	30
Soybean	II	20	nil	10	4.6	10.2	90
		20	3	10	4.0	9.4	85
		20	6	10	3.3 [1]	8.3	80
		20	9	10	2.9 [1]	7.7	76
Herring	I	20	nil	16	2.6	8.1	64
		20	10	16	0.89 [1]	6.3	28

[1] Weight gains significantly lower than those of control animals fed the unheated oil.

[2] Weight gains significantly lower than those of animals fed linseed oil heated only 4 hours.

In table 2 is presented a summary of the average daily gains and feed intakes of animals fed in 6 different tests covering a series of potentially edible oils.

It should be noted that the duration of heating stated is not necessarily the earliest point at which the nutritional value of the oil is damaged. Our experiments, for example, have shown linseed oil heated two hours at polymerizing temperatures, and soybean oil heated three hours, to be as nutritious as the unheated control oils. The intermediate periods between 15 and 30 hours for rapeseed, corn, and peanut oils have not been tested, nor the periods up to 10 hours with herring oil.

TABLE 3

Effect of 10% vs. 20% of heated oil in the diets as measured by daily gains and feed intake ratios of rats

TEST AND OIL FED	OIL IN DIET	DURATION OF HEAT TREATMENT	NO. OF ANIMALS	AVE. DAILY GAIN	AVE. DAILY FEED INTAKE	GAIN/1,000 CALORIES
	%	*hrs.*		*gm*	*gm*	*gm*
Linseed	20	nil	10	3.7	10.1	80
	10	12	10	2.3 [1]	8.2	72
	20	12	10	−0.1 [2]	6.0	4
Soybean	10	nil	10	3.7	10.3	92
	10	6	10	3.5	10.4	87
Soybean	20	nil	10	4.6	10.2	90
	20	6	10	3.3 [1]	8.3	76

[1] Weight gain significantly lower than that of group fed unheated oil.

[2] Weight gain significantly inferior to that of group fed heated oil as 10% of the diet.

That the duration of heating is a factor in the extent of the damage in linseed and soybean oils is clearly indicated by the progressive decrease in weight gains as the heating period was prolonged.

The data presented in table 3 indicate that the diet becomes progressively poorer as the proportion of heated oil in it increases.

When linseed oil, heated 12 hours, was fed as 10% of the diet, the growth resulting over a 4-week period was significantly lower than that of animals fed unheated oil. When the heated oil was included in the diet as 20% by weight, the final

weight of the group was even lower than the starting weight, showing extreme growth inhibition.

When soybean oil was fed as 10% of the diet, oil heated 6 hours appeared as nutritious as the unheated oil. However, when oil treated in the same manner was fed as 20% of the diet, significantly lower gains resulted.

While the results from this series of trials leave no doubt that heat polymerization does adversely affect the nutritive value of the oils examined, the mechanism of this damage was not elucidated. Digestion studies rule out incomplete absorption as a causal factor. It can be postulated that growth inhibition was either the result of some factor in the heated oil toxic to the animals, or that it was a consequence of the destruction by abnormal products of fat polymerization of some necessary dietary component other than fat. To differentiate between these two possibilities as a guide to further investigations, the effect of feeding polymerized linseed oil by dropper was compared to that of feeding the oil mixed into the diet.

Animals fed the oil by dropper were given free access for 16 hours daily to diet III minus the fat. Subsequently, and following three hours of fasting, an amount of oil was administered equal to 10% of the fat-free diet consumed during the preceding 16-hour feeding period. Five hours then elapsed before access to the diet was again permitted.

The results of a 28-day feeding trial conducted in this manner are summarized in table 4.

The method of oil feeding had no effect on the growth of animals receiving unheated oil. However, the growth of animals fed the heated oil by dropper appeared superior to that of animals receiving the heated oil as part of the diet mixture, perhaps because the oil-free diet was more acceptable than the mixture containing the heated fat. However, comparison of gains made in relation to equal caloric intake shows that feed utilization was equally poor in the diets in which heated oil was included, regardless of the method of oil feeding.

We are not prepared at this point to explain why appeti was depressed only when the heated oil was a diet component. However, the observation that growth was depressed eve when the oil was fed apart from the rest of the diet indicate that the deleterious effects exerted by polymerized oils were not incurred through the destruction of some essential non-lipid diet component, but through some oil constituent that acted in a toxic manner directly upon the animals' metabolic pools. This is in accord with the previous observations that the growth depression was intensified as the level of polymerized oil in the diet was increased, and also as the time of heating was lengthened.

TABLE 4

Summary of daily gains, feed intake and gains per 1,000 Calories eaten of animals fed polymerized linseed oil by dropper and as a dietary component

TREATMENT OF OIL	METHOD OF OIL FEEDING	NO. OF ANIMALS	AVE. DAILY GAIN	AVE. DAILY FEED INTAKE	GAIN/1,000 CALORIES
			gm	*gm*	*gm*
Unheated	As a diet component	8	4.2	12.4	87
Unheated	by dropper	8	4.1	12.5	85
Heated 12 hrs.	As a diet component	8	2.7	9.7	71
Heated 12 hrs.	by dropper	8	3.4	12.4	71

It appears logical at this time, therefore, to conclude that the toxic material is some product of polymerization and that its development is related to the unsaturation of the oil. Linseed is the most highly unsaturated of all the oils tested and was the most rapidly susceptible to nutritive damage by heating.

A series of nutritional studies of various fractions of polymerized oils designed to identify the toxic substance or substances will be reported in a subsequent paper.

CONCLUSIONS

From the data reported herein, the following conclusions are warranted:

1. Ingestion of certain oils heat-polymerized at 275°C. has been shown to depress rat growth and the efficiency of feed utilization. This reflects a lowered nutritive value of those oils which varies in severity with the degree of unsaturation of the oil, the length of time of heating each oil, and the level at which the heated oil is incorporated in the diet.

2. The factor or factors responsible for these adverse nutritional effects appear to be present in the oil itself, and act in a manner directly toxic to the animal.

ACKNOWLEDGMENTS

The authors are indebted to a number of persons not connected with this Department, whose collaboration facilitated the performance of this series of experiments.

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The soybean oil was prepared and contributed through the courtesy of Dr. W. D. McFarlane, Canadian Breweries, whose personal interest in these studies has been of much help.

Special chemical facilities were made available by Dr. R. H. Common, Department of Chemistry, Macdonald College, who also assisted in the planning and interpretation of these studies.

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THE NUTRITION OF THE MOUSE

X. STUDIES ON THE UTILIZATION OF HIGH AND MODERATELY LOW PROTEIN DIETS FOR GROWTH IN FOUR STRAINS OF MICE [1]

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FOUR FIGURES

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Tumor growth, whatever its cause, involves the deposition of new tissue and the synthesis of new protein. It may be expected to give rise to measurable alterations in the pattern of nitrogen metabolism of the host. This immediately raises the question of whether the nitrogen metabolism of a tumor-susceptible animal during the period before tumors appear differs from that of a non-susceptible one.

Mice of the highly inbred strains C_{57}, I, A and C_3H maintained in our colony seemed to be particularly suited to the investigation of this question since the first two strains show a low incidence and the last two a high incidence of spontaneous tumors. Previous observations (Fenton and Cowgill, '47; Fenton, Cowgill, Stone and Justice, '50) have shown significant strain differences to exist with respect to the requirements of certain vitamins for growth. It seemed possible that there might also be differences with respect to

[1] This investigation was supported by research grants from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council; the National Cancer Institute, U. S. Public Health Service; and the Anna Fuller Fund.

Vitamins of the B complex were obtained through the courtesy of Hoffmann-LaRoche, Inc. Folic acid was furnished by the Lederle Laboratories Division, American Cyanamid Company.

the utilization of protein for growth. While a considerable amount of work has been done on the amino acid requirements of mice (Bauer and Berg, '43; Sauberlich and Baumann, '46; Pearce et al., '47; Maddy and Elvehjem, '49), relatively little is known concerning general nitrogen metabolism in this species. The principal contributions have been made by Bosshardt and his associates ('46a, b; '48).

METHODS

Mice of 4 strains (C_{57}, I, A and C_3H) [2] were weaned at 21 or 22 days of age and placed on a diet containing 10% casein (diet 241, table 1). This level of dietary protein was chosen because Bosshardt et al. ('46b) have found it to yield maximum protein efficiency ratios. The diet was fed ad libitum, but extensive precautions were taken to prevent scattering of food and contamination with urine and feces. This aim was accomplished in most instances. Food consumption and weight gains were recorded daily for a 15-day period. Thereafter the animals were weighed only every third day. Similar studies were carried out with a diet containing 30% casein (diet 223, table 1).

Other groups of mice of the 4 strains were fed a high protein diet (diet 224, table 1). In this experiment it was difficult to obtain an accurate measure of food consumption, since the animals were extremely untidy. When it was found that the 90% casein diet did not support normal growth, efforts were made to supplement or modify the ration. Among the supplements used were sodium bicarbonate (diets 230 and 232). In another experiment the casein was replaced largely by lactalbumin (diet 242). Since high protein diets are known to increase the requirements for certain vitamins, diets were prepared identical with 224 but containing respectively 5 and 18 times the amount of the B vitamins.

In the course of certain unpublished experiments it was decided to devise a salt mixture which would be relatively

[2] Bred in our colony and originally obtained from Dr. Leonel C. Strong, Yale University.

TABLE 1

Composition of diets

CONSTITUENTS	DIET						
	223	224	229	237	241	242	244
	%	%	%	%	%	%	%
Casein [1]	30	90	5	5	10	5	90
Lactalbumin	..	..	..	..	..	85	..
Egg albumin	..	..	85	..	..	..	..
Blood fibrin	..	..	..	85	..	..	..
Sucrose	..	..	..	..	80	..	..
Dextrin	60	..	..	..	..	..	..
Corn oil [2]	5	5	5	5	5	5	5
Salt mixture A [3]	5	5	5	5	5	5	..
U.S.P. salts no. 2	..	..	..	..	..	..	5

Diet 230: 100 gm diet 224 plus 10 gm $NaHCO_3$.

Diet 232: 100 gm diet 224 plus 3 gm $NaHCO_3$.

Diet 233: identical with 224 but containing 18 times as much of all the B vitamins.

Diet 236: identical with 224 but containing 5 times as much of all the B vitamins.

[1] Labco, vitamin-free. Vitamins of the B complex were added in the following amounts per 100 gm of diet: thiamine 1 mg, pyridoxine 1 mg, riboflavin 2 mg, nicotinic acid 5 mg, pantothenic acid 10 mg, choline chloride 150 mg, biotin 20 μg, folic acid 500 μg.

[2] Mazola. Fat-soluble vitamins were added in the following amounts per 100 gm of diet: A 1,000 I.U., D 100 I.U., α-tocopherol 5 mg, 2-methyl-naphthoquinone 1 mg.

[3] For composition see table 2.

TABLE 2

Composition of salt mixture A

SALT MIXTURE A		TRACE MIXTURE [1]	
Constituents	*Amount gm*	*Constituents*	*Amount gm*
$CaHPO_4$	1,200		
K_2HPO_4	400	$CoCl_2 \cdot 6H_2O$	0.5
NaCl	400	$CuBO_4$	0.5
$MgSO_4$	100	$AlF_3 \cdot xH_2O$	0.5
Ferric citrate	50	$MnSO_4 \cdot H_2O$	5.5
ZnI_2	0.5		
Trace mixture	7		

[1] The trace mixture may be prepared in quantity.

simple to prepare (salt mixture A, table 2). Instead of adding separately the salts of Al, Cu, Co, Zn, I, F, and B, compounds such as ZnI_2, AlF_3, and $CuBO_4$ were employed. Salt mixture A was found to support growth as well as certain other salts in common use. Diet 244 is identical with 224 but contains commercial U.S.P. Salts no. 2. It was used specifically to provide a comparison of Salts A with U.S.P. Salts 2 when used in conjunction with a high protein diet.

A total of 250 mice were used in these experiments.

RESULTS

The protein efficiency ratios obtained with the 4 strains of mice on the 10% casein diet over a 15-day period are shown in table 3. Contamination of the food cups with

TABLE 3

Protein efficiency ratios obtained with 4 strains of mice

STRAIN	WEIGHT GAIN	FOOD CONSUMPTION [1]	PROTEIN EFFICIENCY RATIO
	gm	*gm*	
C_3H	7.4	51.7	1.43
A	4.8	41.6	1.13
C_{57}	3.1	30.7	0.98
I	2.3	34.0	0.65

[1] Diet 241.

urine proved to be a problem with only a few mice of the A strain. In consequence the protein efficiency ratio cited for this strain may be just a trifle too high. The growth curves of the 4 strains for the 30-day period following weaning are shown in figure 1.

Figure 2 shows the growth curves of all 4 strains of mice on diet 224 (90% casein). In this experiment mice of the I strain were found to gain weight at a considerably slower rate than animals of the other three strains. On diets containing 30% casein a similar but less pronounced difference

in response was also observed. It is significant that all mice lost weight during the first few days on the high protein diet. This is not usually observed with adequate diets of

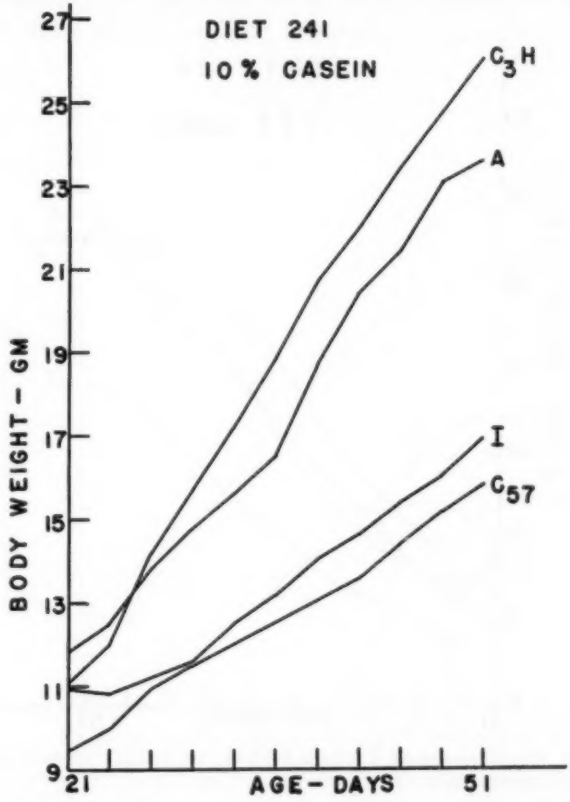

Fig. 1 Growth curves of 4 strains of mice fed diet 241 (10% casein) from weaning to age 51 days.

moderate protein content. Food consumption was appreciably reduced on the 90% protein diet. In figure 3 are shown the effects of supplementing the high casein diet with $NaHCO_3$ and of replacing most of the casein with lactalbumin. All

high protein diets were found to aggravate the normally aggressive disposition of the I strain mice.

When the weight gains of the 4 strains of mice were plotted against the casein content of the diet (fig. 4), dichotomy was

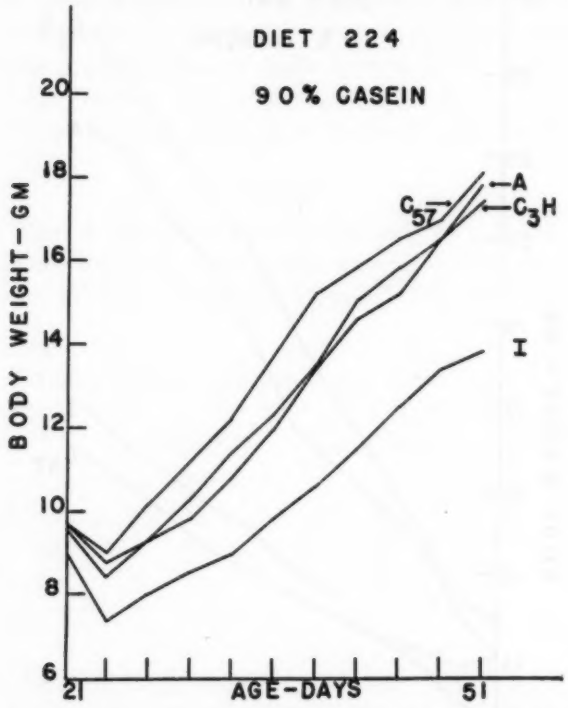

Fig. 2 Growth curves of 4 strains of mice fed diet 224 (90% casein) from weaning to age 51 days.

again observed. C_3H and A strain mice showed essentially the same degree of weight gain on 10% and on 30% casein diets, while I and C_{57} mice gained weight maximally with the 30% protein diets.

Results with diet 244 indicated that salt mixture A, designed for simplicity of preparation, when used in conjunc-

tion with a high protein diet supports growth as well as U.S.P. Salts no. 2. The use of our new salt mixture in other diets has invariably given results comparable to those obtained with several salt preparations in common use.

Diets 229 and 237 resulted in poor growth.

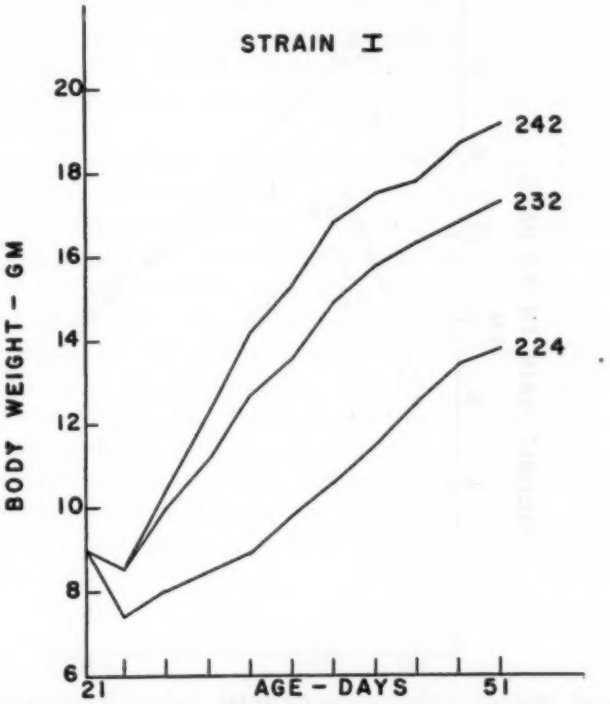

Fig. 3 Growth curves of I strain mice fed diets 224 (90% casein), 232 (90% casein with 3% $NaHCO_3$) and 242 (85% lactalbumin and 5% casein) from weaning to age 51 days.

DISCUSSION

The great spread in food consumption, weight gain and protein efficiency ratio among the 4 strains of mice (table 3) emphasizes the need of using inbred strains and of stating precisely the strain and source of animals used in studies

on protein efficiency. The protein efficiency ratios obtained in this study were lower than those observed by Bosshardt et al. ('46a) and may be attributed to the fact that they used animals weaned at 15 or 16 days of age, while ours were

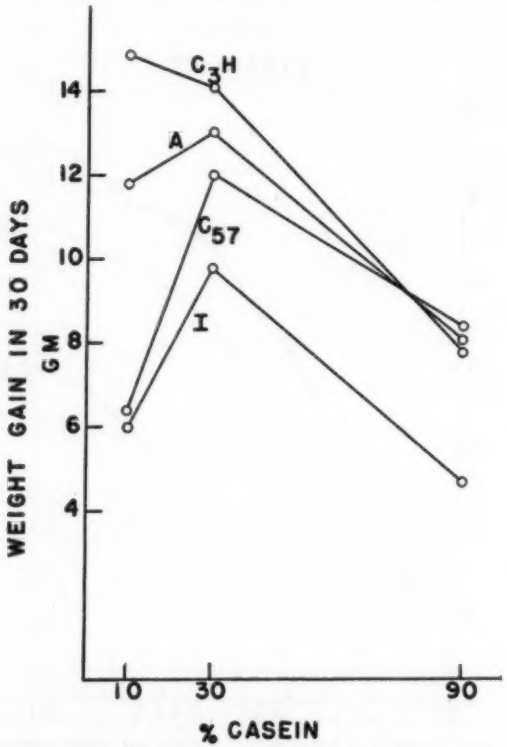

Fig. 4 Total weight gains, from weaning to age 51 days, of 4 strains of mice plotted against the protein content of the diet.

weaned at 21 or 22 days. Over the 15-day period employed in this study the weight gains of A and C_{57} strain mice were not markedly different. When the growth curves are plotted over a 30-day period (fig. 1), the differences between the A and C_{57} strains become more apparent. The retarded growth

of the C_{57} animals is the more dramatic when one takes cognizance of the fact that on more nearly adequate diets they usually show the most rapid rate of weight gain.

That the growth-retarding effect of the 90% casein diet is not due to the absence of carbohydrate has been proved in experiments to be reported elsewhere. The fact that $NaHCO_3$ in part overcame this retardation suggests that acidosis may have played a role. This point as well as other possible explanations are at present under more thorough examination.

The curves plotted in figure 4 reveal the existence of strain differences with respect to the ability to utilize for growth diets of varying protein content. Bosshardt et al. ('46a) have shown that under the conditions of their experiments maximum growth was obtained with diets containing between 15 and 20% casein. Growth was distinctly less with 10% casein diets. This is in agreement with our findings with the C_{57} and I strains (tumor-resistant). The C_3H and A strains, (tumor-susceptible), however, grew as well or nearly as well on the 10% as on the 30% casein diet.

It is clear, then, that strain differences do exist with respect to the utilization of protein and that this observation differentiates the tumor-susceptible from the tumor-resistant strains in our colony.

SUMMARY

1. A wide range of protein efficiency ratios was observed when mice of 4 highly inbred strains were studied under identical conditions.

2. Mice of the C_3H and A strains grew considerably more rapidly on a diet containing 10% casein than did mice of the C_{57} and I strains.

3. The growth rates of all 4 strains studied were reduced by feeding a 90% casein diet.

4. The addition of $NaHCO_3$ or the substitution of lactalbumin for casein gave improved growth.

5. Total weight gains of C_3H and A strain mice over a 30-day period following weaning were essentially the same whether the diet contained 10% or 30% casein.

6. Mice of the C_{57} and I strains gained more weight on a 30% than on a 10% casein diet.

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VITAMIN A AND CAROTENE ABSORPTION IN THE GUINEA PIG[1]

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The pathway of vitamin A and carotene absorption has long been under discussion. Reports have varied as to whether vitamin A is absorbed through the lymphatics or whether the portal system is also involved.

Drummond, Bell and Palmer ('35), working with humans, and Clausen ('43) reporting on the work of McCoord, Breese and Baum on dogs, demonstrated that oral administration of vitamin A affords increased levels of the vitamin in the lymph collected from the thoracic duct. Popper and Volk ('44) observed typical vitamin A fluorescence in the lacteals following dosage of rats with the vitamin. Radice and Herriaz ('47) confirmed the results of Popper and claimed they observed this typical fluorescence in the portal blood of rats. Eden and Sellers ('49), however, using bullocks, sheep, and rats, were unable to demonstrate vitamin A absorption through the portal system when blood was collected from one to 24 hours after supplementation. It was suggested, however, that since portal circulation is rapid, perhaps portal absorption occurred prior to the collection of their samples.

The present report includes a study of the absorption of vitamin A and carotene in the guinea pig, and an estimation of the rate and time necessary for maximum absorption of the vitamin into the mesenteric lymphatics.

[1] Scientific paper 937, Washington Agricultural Experiment Stations, Institute of Agricultural Sciences, The State College of Washington, Pullman.

EXPERIMENTAL PROCEDURES

Guinea pigs of the English variety, interbred with the same males, were obtained from one supply house and fed the stock colony diet for a few weeks prior to the beginning of the experiment.

Animals were selected so that they weighed from 500 to 650 gm. At this weight the animals were not too fat to make mesentery collection impractical.

The animals were fasted 24 hours prior to feeding the vitamin or carotene supplement. The supplements consisted of: (1) 8.8 mg of crystalline vitamin A alcohol in 20 ml of oil.[2] To this, 0.1 gm of alpha-tocopherol was added to prevent oxidation. This yielded a supplement containing approximately 2,000 I.U. of vitamin A per milliliter; (2) 28 mg of crystalline carotene (10% alpha, 90% beta) in 20 ml oil.[3] One-tenth of 1 gm of alpha-tocopherol was also added to this solution. This yielded approximately 2,000 I.U. of beta carotene per milliliter of solution.

Following the forced feeding with 1 ml of either supplement, the animal was sacrificed with excess ether after the required time had elapsed.

A 5-ml syringe with a 25-gauge needle was used to collect the blood samples. The portal vein was punctured at a point half way between the mesentery and the liver. The blood from the dorsal aorta was taken at a point a short distance below the junction of the right and left aortic arches. These samples of blood were allowed to stand in the refrigerator until the serum separated.

The mesenteric lymphatics adjoining the duodenum, jejunum, and ileum were carefully removed, avoiding all major blood vessels and fat depots. The mesentery adjoining the colon was avoided because Barrick, Andrews and Ballard

[2] Mazola.

[3] See footnote 2.

('48) have shown that most of the vitamin A administered is absorbed through the small intestine.

The animals were divided into groups of 6. The groups consisted of: (1) a control group to which no supplement was given but which was fasted the full 24 hours; (2) several groups of animals fasted 24 hours and then given 1 ml of the vitamin A supplement. Each group was sacrificed when the allotted time after supplementation had elapsed. The time factors for these groups were: one-half hour, 45 minutes, one hour, one and one-half hours, and two hours; (3) several groups of animals fasted 24 hours and then force-fed 1 ml of carotene supplement. Each group was sacrificed when the allotted time after supplementation had elapsed. The time factors for these groups were: one-half hour, one hour, one and one-half hours, and two hours.

These animals were analyzed individually after sacrifice and collection of the blood and mesenteric lymphatics.

The Bessey, Lowry, Brock and Lopez ('46) microspectrophotometric method for blood analysis was used to determine the blood carotene and vitamin A levels. Readings were taken on the Beckman spectrophotometer at 460 mμ for carotene and 328 mμ for vitamin A. The micro attachments were used, and slit widths of 0.15 mm for carotene and 1.20 mm for vitamin A were set for the entire experiment.

The following modification of the Bessey et al. method was used to determine the vitamin A and carotene in the mesenteric lymphatics. The mesenteric tissue was homogenized with water in the Potter and Elvehjem ('36) homogenizer. This homogenate was further diluted to a volume of 50 ml. This solution was saponified with 50 ml of 1 N KOH in 90% ethyl alcohol and heated in a water bath at 60°C. for 20 minutes. The mixture was then cooled and centrifuged slowly to remove any protein precipitate.

Two volumes of the saponified solution were extracted with one volume of kerosene-xylene in the same manner in which blood is extracted in the original method.

RESULTS

Table 1 shows that maximum vitamin A absorption into the mesenteric lymphatics occurred at one hour when vitamin A was fed. Statistical analysis using "t" scores (table 2) showed that in one-half hour a statistically significant rise in the mesenteric lymphatic vitamin A levels occurred. This level rose slowly until a peak was reached at one hour. A statistically significant difference between the one hour and

TABLE 1

Average[1] vitamin A levels of the portal blood, dorsal aorta blood, and mesenteric lymphatics

TIME	MESENTERIC LYMPHATICS	PORTAL	DORSAL AORTA
	μg	μg %	μg %
	No supplement		
	3.74 ± 0.50	28.66 ± 0.78	29.91 ± 0.91
	Vitamin A supplement		
½ Hour	6.92 ± 0.44	28.89 ± 1.49	32.33 ± 1.83
¾ Hour	7.06 ± 0.16	28.55 ± 1.04	32.63 ± 1.41
1 Hour	7.22 ± 0.70	29.37 ± 0.88	34.94 ± 1.31
1½ Hours	3.30 ± 0.30	28.93 ± 0.79	33.52 ± 0.83
2 Hours	3.82 ± 0.48	29.11 ± 0.58	37.96 ± 0.76
	Carotene supplement		
½ Hour	3.54 ± 0.34	32.22 ± 1.40	32.59 ± 0.84
1 Hour	10.51 ± 0.57	31.38 ± 0.87	35.19 ± 1.84
1½ Hours	4.49 ± 0.55	30.35 ± 0.78	34.66 ± 0.79
2 Hours	2.79 ± 0.32	30.63 ± 0.99	35.47 ± 0.87

[1] Including the standard error of the mean as calculated from the formula given in Snedecor ('46).

one and one-half hour levels clearly demonstrated that the peak was at one hour. This high level dropped off rapidly and reached the normal fasting level in less than two hours after supplementation.

As this drop occurred, a statistically significant rise in the vitamin A blood levels of the dorsal aorta was found. The highest dorsal aorta blood level was found at two hours' absorption time and proved to be statistically different from the levels obtained in one and one-half hours. However, it is

possible that this level may rise even higher in a longer period of time.

There was no significant change in the portal blood, dorsal aorta blood, or mesenteric lymphatic carotene levels after supplementation with carotene. This seems to indicate that carotene, as such, is not absorbed through the portal or lymphatic systems. Statistical analysis using "t" scores (table 2)

TABLE 2

Statistical analysis of differences between groups

ABSORPTION COMPARED	VITAMIN A LEVELS WITH VITAMIN A SUPPLEMENT		VITAMIN A LEVELS WITH CAROTENE SUPPLEMENT	
	Mean difference	"t" [1]	Mean difference	"t" [1]
Mesenteric lymphatics				
Control vs. ½ hour	3.18	15.16 [2]	0.09	0.15
Control vs. ¾ hour	3.32	6.38 [2]	...	...
Control vs. 1 hour	3.48	4.08 [2]	6.77	8.99 [2]
Control vs. 1½ hours	0.44	0.76	0.75	1.02
Control vs. 2 hours	0.08	0.12	0.95	1.62
1 hour vs. 1½ hours	3.92	5.17 [2]	6.02	7.64 [2]
½ hour vs. 1 hour	0.30	0.49	6.86	10.35 [2]
Dorsal aorta				
Control vs. ½ hour	2.42	1.12	2.68	2.08
Control vs. 1 hour	5.03	3.14 [2]	5.28	2.58 [2]
Control vs. 1½ hours	3.61	1.93	4.75	3.92 [2]
Control vs. 2 hours	8.05	6.76 [2]	5.56	4.50 [2]
1½ hours vs. 2 hours	4.44	3.94 [2]	0.81	0.70

[1] Calculated from Fisher's formula (Snedecor, '46).

[2] Significant at the 1% level.

[3] Significant at the 5% level.

reveals, however, that significant changes in vitamin A dorsal aorta blood and mesenteric lymphatic levels occurred when carotene was fed.

This agrees with the work of Goodwin and Gregory ('48), who showed that in rabbits, sheep and goats, the presence of carotene could not be demonstrated in the portal or systemic blood after massive oral doses of carotene. However. these

workers demonstrated increased vitamin A levels in the lymph of goats resulting from the feeding of carotene.

The carotene levels of guinea pigs' blood were found to be extremely low, being between 2 and 4 μg %. The mesenteric lymphatic levels were practically zero, being under 1 μg.

One hour was found to be the time of maximum absorption into the mesenteric lymphatics of the vitamin A formed from the carotene fed. This is clearly borne out by the fact that the one-hour levels were statistically different from those obtained in one-half hour and one and one-half hours.

The vitamin A blood levels of the dorsal aorta rose until they reached a peak at two hours when carotene was fed. However, it is possible that these levels may rise even higher in a longer period of time.

There was no significant change in the portal vitamin A blood levels between groups when either vitamin A or carotene was administered. This would indicate that the major pathway of absorption for preformed vitamin A and vitamin A formed from carotene is through the lymphatic system rather than the portal system of the guinea pig.

DISCUSSION

Vitamin A alcohol appears to follow the path of fat absorption in the guinea pig, in that it is absorbed principally through the lacteals. It is reasonable to postulate that vitamin A from the lacteals empties into the thoracic duct, as fat does, and then enters the blood stream at the jugular vein. Since this vitamin A is found again in the dorsal aorta, it would seem likely that it is pumped through the heart into the general circulation.

The data indicate that in the guinea pig vitamin A absorption from the intestine is completed in less than two hours when oil [4] is used as a solvent. At two hours, the vitamin A of the mesenteric lymphatics was at the same level as when no supplement was given.

[4] See footnote 2, page 452.

It is interesting to note that the vitamin A formed from carotene was not absorbed into the lymphatics as quickly as preformed vitamin A. It may be possible that some time is required to affect the conversion of carotene to vitamin A.

Although the vitamin A values of the portal blood showed no statistically significant increase after carotene supplementation, it is interesting to note that all portal blood values for vitamin A were higher after carotene supplementation. It is possible that there may be some absorption of vitamin A by way of the portal system after carotene to vitamin A conversion. Statistics may fail to support this statement because of the rapid portal circulation or the slow conversion of carotene to vitamin A, or both. However, the major pathway of absorption in all cases appears to be by way of the lymphatics.

Numerous workers have concluded that carotene to vitamin A conversion occurs in the intestinal wall. This evidence is substantiated further in this study with the guinea pig by the fact that very little carotene was present in the mesenteric lymphatics after carotene supplementation, while the vitamin A levels rose significantly.

Eden and Sellers ('49) suggested that portal absorption may possibly occur a short time after supplementation because portal circulation is rapid. However, portal absorption of vitamin A alcohol could not be demonstrated in one-half hour in this laboratory. This would seem to indicate that the lacteals are the principal pathway of vitamin A absorption, and that carotene as such is not absorbed.

SUMMARY

Fasted guinea pigs were supplemented with either carotene or vitamin A (approximately 2,000 I.U.) and then sacrificed in one-half hour, 45 minutes, one hour, one and one-half hours, or two hours. The levels of vitamin A and carotene in the portal blood, systemic blood, and mesenteric lymphatics were determined.

1. Dosage with either vitamin A or carotene affected a rise in the vitamin A levels of the mesenteric lymphatics and the dorsal aorta blood.

2. Carotene absorption was found to be practically negligible when carotene was fed, but significant increases in vitamin A dorsal aorta blood and mesenteric lymphatic levels occurred.

3. The time of maximum absorption of vitamin A itself or vitamin A formed from the carotene fed, was at one hour after supplementation.

4. Portal absorption of either vitamin A or carotene could not be statistically demonstrated.

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